DESCRIPTION

METHOD OF PRODUCING RECOMBINANT PROTEIN IN BACTERIUM BELONGING TO GENUS *RHODOCOCCUS*

Technical Field

The present invention relates to an expression vector capable of expressing a foreign gene in a bacterium belonging to the genus *Rhodococcus*.

The present invention also relates to an inducible expression vector and a constitutive expression vector capable of expressing a recombinant protein in a host cell, and to a method of using any of the vectors to express a recombinant protein. The present invention further relates to a method of simultaneously expressing several genes encoded on different vectors in the cell of a bacterium belonging to the genus *Rhodococcus*.

Background Art

Currently, an expression system using *Escherichia coli* as a host is widely used for preparing a large amount of a recombinant protein derived from a eukaryote. This is because the system is readily handled and its research progresses most (Weickert et al., Curr. Opin. Biotechnol. *7*: 494-499 [1996]).

On the other hand, the present inventors have previously demonstrated that *Rhodococcus erythropolis* can also be used as a host for recombinant protein production (JP Patent Publication (Kokai) No. 2004-73032A and Japanese Patent Application No. 2002-235008). *R. erythropolis* is one of *Actinobacteria* capable of proliferation at temperatures ranging from 4°C to 35°C. The biggest feature of an expression system that uses this bacterium as a host is in that a recombinant protein can be produced at temperatures not higher than 10°C such as 4°C. In systems that employ other hosts including *Escherichia coli*, bacteria belonging to the genus *Bacillus*, yeasts, and Sf9 insect cells (Cereghino and Cregg, Curr. Opin. Biotechnol. *10* 422-427 [1999]; and Miller, Curr. Opin. Genet. Dev. *3* 97-101 [1993]), recombinant protein production at temperatures not higher than 10°C is quite

difficult. Recombinant protein production at temperatures not higher than 10°C has allowed the production of proteins difficult to produce until then, for example, proteins inhibiting the proliferation of host cells, proteins getting insoluble at temperatures around 30°C, and proteins derived from organisms adapting to low temperatures.

The present inventors have constructed a group of expression vectors for a bacterium belonging to the genus *Rhodococcus* called pTip vectors and have used them in recombinant protein production (JP Patent Publication (Kokai) No. 2004-73032A and Japanese Patent Application No. 2002-235008). These vectors contain the promoter of a *TipA* gene whose expression is induced by antibiotic thiostrepton and also contain a multiple-cloning site (MCS) for cloning a foreign gene (gene to be expressed), located downstream of the promoter. Thus, the pTip vectors are thiostrepton-inducible expression vectors. In bacteria belonging to the genus *Rhodococcus* that are transformed with these expression vectors, foreign protein production is induced only when thiostrepton is added to a culture solution.

Non-Patent Document 1

Weickert et al., Curr. Opin. Biotechnol. *7*: 494-499 [1996]

Non-Patent Document 2

Cereghino and Cregg, Curr. Opin. Biotechnol. *10* 422-427 [1999]

Non-Patent Document 3

Miller, Curr. Opin. Genet. Dev. *3* 97-101 [1993]

Disclosure of the Invention

Although the present inventors, as described above, have constructed a group of expression vectors for a bacterium belonging to the genus *Rhodococcus* called pTip vectors and have used them in recombinant protein production, two things have been left for development.

First, several expression vectors containing the same autonomous replication origin but different foreign genes had difficulty in simultaneously and stably coexisting in a bacterium belonging to the genus *Rhodococcus*, because the DNA regions of all of the pTip vectors necessary for autonomous replication in the bacterium belonging to the genus *Rhodococcus*

were derived from a single endogenous plasmid. This is due to a phenomenon called plasmid incompatibility in which heterologous plasmids having the same autonomous replication origin can not coexist in a bacterium, and there has been a report on this phenomenon for many bacteria (Novick, Microbiol. Rev. *51* 381-395 [1987]). If heterologous plasmids can be allowed to coexist in a single bacterial cell, several recombinant proteins can simultaneously be produced. For example, a protein complex called 20S proteasome is composed of two polypeptides, an α subunit and a β subunit. When functional 20S proteasome complex is produced as a recombinant, these two polypeptides must be coexpressed. The coexpression of two polypeptides in a single cell can also be achieved by introducing several foreign genes into a single expression vector. However, such an approach utilizes a large-sized vector and a complicated cloning step for restriction enzyme site reasons and as such, is inconvenient in most cases. An established coexpression system of recombinant proteins in a bacterium belonging to the genus *Rhodococcus* by the use of several expression vectors is described in WO02/055709.

Second, in spite of the fact that a key tool in research for bacteria belonging to the genus *Rhodococcus* is not only inducible expression vectors but constitutive expression vector, the constitutive expression vector was undeveloped. Those using a mutated nitrile hydratase gene promoter (JP Patent Publication (Kokai) No. 9-28382A (1997) and JP Patent Publication (Kokai) No. 10-248578A (1998)) and using an *rrn* promoter are known as constitutive expression vectors in known bacteria belonging to the genus *Rhodococcus* (Matsui et al., Curr. Microbiol. *45* 240-244 [2002]).

Among bacteria belonging to the genus *Rhodococcus*, many strains that decompose various persistent compounds such as PCB (polychlorinated biphenyl) and pesticides are known (bioremediation) (Bell et al., J. Appl. Microbiol. *85* 195-210 [1998]), and a certain strain is known to accumulate useful compounds such as acrylamide in the bacterial cell and is already utilized in industrial production (bioprocess and bioreactor) (Yamada et al., Biosci. Biotech. Biochem. 60 1391-1400 [1996]). Thus, if the above-described two things to be improved were overcome, the availability of an expression vector for a bacterium belonging to

the genus *Rhodococcus* would be increased not only in recombinant protein production but in research for bioremediation and bioprocess.

The solution to a problem of plasmid incompatibility requires newly separating and utilizing an equivalent sequence different from the sequences of the DNA regions used in the pTip vectors previously constructed by the present inventors, which are necessary for autonomous replication in a bacterium belonging to the genus *Rhodococcus*. The minimal *RepAB* gene-containing region (1.9 kilobase pairs; hereinafter, abbreviated to kb), necessary for autonomous replication, of an endogenous plasmid pRE2895 (5.4 kb) separated from a *R. erythropolis* strain JCM2895 has been used in all of the pTip vectors. Thus, the present inventors have decided to construct novel expression vectors by separating endogenous plasmids having different DNA sequences from other *R. erythropolis* strains. In the construction of the pTip vectors, only a tetracycline resistance gene has been developed as a selection marker for the transformant of a bacterium belonging to the genus *Rhodococcus*. However, the transformation of the bacterium belonging to the genus *Rhodococcus* with several plasmids requires other resistence genes. The present inventors have found that a *R. erythropolis* strain DSM313 is resistant to chloramphenicol and have decided to separate and utilize a gene that imparts resistance.

For developing a constitutive expression vector, the present inventors have further decided to introduce a mutation into the *TipA* gene promoter to produce a mutant that allows the constitutive expression, that is thiostrepton-independent expression of a foreign gene.

In this way, in addition to the pTip vectors having the region of the pRE2895 necessary for autonomous replication and the inducible *TipA* gene promoter, the present inventors have newly constructed: a vector having a different DNA region necessary for autonomous replication, which is a vector having the *TipA* gene promoter and allowing inducible expression; a vector having a different DNA region necessary for autonomous replication from those of the pTip vectors, which is a vector having a promoter where a mutation is introduced into the *TipA* gene promoter and allowing constitutive expression; and a vector having the same DNA region necessary for autonomous replication as those of the pTip vectors and a promoter where a mutation is introduced into the *TipA* gene promoter and allowing

constitutive expression. Of these vectors, two types of vectors having different DNA regions necessary for autonomous replication and containing genes encoding foreign proteins different from each other can be used to cotransform one host. In addition, the different foreign proteins can simultaneously be coexpressed in the cotransformed host.

That is, the present invention is as follows:

[1] DNA comprising a nucleotide sequence of a mutated *TipA* gene promoter where a mutation is introduced into a -10 region sequence of a *TipA* gene promoter, the mutated *TipA* gene promoter capable of thiostrepton-independent and constitutive expression of a gene located downstream thereof;

[2] The DNA of [1], wherein the mutation in the -10 region sequence is a mutation of a CAGCGT sequence to a TATAAT sequence;

[3] The DNA of [2], having a nucleotide sequence represented by SEQ ID NO: 107;

[4] A constitutive expression vector for a bacterium belonging to the genus *Rhodococcus* comprising: a promoter sequence for the constitutive expression of a foreign gene, the promoter sequence being a nucleotide sequence of DNA of any one of [1] to [3]; a ribosome-binding site sequence located downstream of the promoter sequence; and a multiple-cloning site sequence capable of incorporating a foreign gene therein, located downstream of the ribosome-binding site sequence;

[5] The constitutive expression vector for a bacterium belonging to the genus *Rhodococcus* of [4], wherein the vector is selected from the group consisting of pNit-RT1 having a nucleotide sequence represented by SEQ ID NO: 101, pNit-RT2 having a nucleotide sequence represented by SEQ ID NO: 102, pNit-RC1 having a nucleotide sequence represented by SEQ ID NO: 105, pNit-RC2 having a nucleotide sequence represented by SEQ ID NO: 106, pNit-QT1 having a nucleotide sequence represented by SEQ ID NO: 99, pNit-QT2 having a nucleotide sequence represented by SEQ ID NO: 100, pNit-QC1 having a nucleotide sequence represented by SEQ ID NO: 103, and pNit-QC2 having a nucleotide sequence represented by SEQ ID NO: 104;

[6] The expression vector of [4] or [5], wherein the bacterium belonging to the genus *Rhodococcus* is selected from the group consisting of *R. erythropolis*, *R. fascians*, and *R. opacus*;

[7] The expression vector of any one of [4] to [6], wherein the vector further comprises a DNA region necessary for the autonomous replication of a plasmid for *Escherichia coli*, and being capable of replication in *Escherichia coli*;

[8] A transformant comprising an expression vector of any one of [4] to [7]; and

[9] A method of producing a recombinant protein at the temperatures ranging from 4°C to 35°C by using an expression vector of any one of [4] to [7].

In addition, the present invention is as follows:

[10] A circular plasmid that replicates in the rolling-circle mode, isolated from a bacterium belonging to the genus *Rhodococcus*;

[11] The circular plasmid of [10] isolated from a bacterium belonging to the genus *Rhodococcus*, having a *Rep* gene, a double-stranded origin (DSO), and a single-stranded origin (SSO) essential for the rolling circle mode of replication;

[12] The circular plasmid of [11], wherein a nucleotide sequence of DNA essential for the rolling circle mode of replication is a nucleotide sequence at positions 3845 to 5849 in a nucleotide sequence represented by SEQ ID NO: 90;

[13] The plasmid of any one of [10] to [12], having DNA having a nucleotide sequence represented by SEQ ID NO: 90 or DNA that hybridizes under stringent conditions to DNA having a complementary sequence to the DNA whose nucleotide sequence is represented by SEQ ID NO: 90;

[14] A transformant comprising a circular plasmid of any one of [10] to [12];

[15] An expression vector that replicates in the rolling-circle mode, being capable of expression a foreign gene under a temperature ranging from 4°C to 35°C in a bacterium belonging to the genus *Rhodococcus*;

[16] The expression vector of [15] having a *Rep* gene, a double-stranded origin (DSO), and a single-stranded origin (SSO) essential for the rolling-circle mode of replication, which

originates from an endogenous plasmid isolated from a bacterium belonging to the genus *Rhodococcus*;

[17] The expression vector of [16], wherein a nucleotide sequence of DNA essential for the rolling circle mode of replication is a nucleotide sequence at positions 3845 to 5849 in a nucleotide sequence represented by SEQ ID NO: 90;

[18] The expression vector of any one of [15] to [17], comprising an inducible promoter sequence for inducing a foreign gene expression, a ribosome-binding site sequence located downstream of the promoter sequence, and a multiple-cloning site sequence capable of incorporating a foreign gene therein, located downstream of the ribosome-binding site sequence;

[19] The expression vector of [18], wherein the inducible promoter for inducing expression is a *TipA* gene promoter and an inducer is thiostrepton;

[20] The expression vector of [4], wherein a nucleotide sequence of the promoter consists of a nucleotide sequence of DNA of any one of [1] to [3];

[21] The inducible expression vector for a bacterium belonging to the genus *Rhodococcus* of any one of [15] to [19], wherein the vector is selected from the group consisting of pTip-RT1 having a nucleotide sequence represented by SEQ ID NO: 93, pTip-RT2 having a nucleotide sequence represented by SEQ ID NO: 94, pTip-RC1 having a nucleotide sequence represented by SEQ ID NO: 97, and pTip-RC2 having a nucleotide sequence represented by SEQ ID NO: 98;

[22] An expression vector capable of expressing a foreign gence consititutively under temperature ranging from 4°C to 35°C in a bacterium belonging to the genus *Rhodococcus*, comprising a plasmid pRE2895-derived DNA sequence necessary for the autonomous replication of a plasmid in a bacterium belonging to the genus *Rhodococcus* and a promoter sequence DNA of any one of [1] to [3];

[23] The expression vector of [22], wherein the plasmid pRE2895-derived DNA sequence necessary for the autonomous replication of a plasmid in a bacterium belonging to the genus *Rhodococcus* is a DNA sequence of a 1.9-kb region containing *RepA* and *RepB* genes;

[24] The expression vector of [22] or [23], further comprising a ribosome-binding site sequence located downstream of the constitutive promoter sequence and a multiple-cloning site sequence capable of incorporating a foreign gene therein, located downstream of the ribosome-binding site sequence;

[25] The constitutive expression vector for a bacterium belonging to the genus *Rhodococcus* of any one of [22] to [24], wherein the vector is selected from the group consisting of pNit-QT1 having a nucleotide sequence represented by SEQ ID NO: 99, pNit-QT2 having a nucleotide sequence represented by SEQ ID NO: 100, pNit-QC1 having a nucleotide sequence represented by SEQ ID NO: 103, and pNit-QC2 having a nucleotide sequence represented by SEQ ID NO: 104;

[26] A bacterium belonging to the genus *Rhodococcus* having at least two types of plasmids:

(i) which are all derived from a bacterium belonging to the genus Rhodococcus,

(ii) which do not cause plasmid incompatibility with each other,

(iii) one of which has a DNA sequence involved in rolling-circle mode of replication and one of the other plasmids has DNA sequence derived from pRE2895 for autonomous replication;

[27] A bacterium belonging to the genus *Rhodococcus* having at least two types of expression plasmid vectors:

(i) which are all derived from a bacterium belonging to the genus Rhodococcus,

(ii) which do not cause plasmid incompatibility with each other,

(iii) which carry genes encoding foreign proteins to be coexpressed under a temperature ranging from 4°C to 35°C,

(iv) one of which has a DNA sequence involved in rolling-circle mode of replication and one of the other plasmids has DNA sequence derived from pRE2895 for autonomous replication;

[28] The bacterium belonging to the genus *Rhodococcus* of [27], wherein all of the two types of plasmid vectors respectively comprise a promoter sequence for foreign protein production, a ribosome-binding site sequence located downstream of the promoter sequence, and a multiple-cloning site sequence capable of incorporating a foreign gene therein, located downstream of the ribosome-binding site sequence;

[29] The bacterium belonging to the genus *Rhodococcus* of [27] or [28], wherein one of the at least two types of plasmid vectors is a vector of any one of [4], [5], [15] to [19], [20], and [21] and another is a vector of any one of [22] to [25] or a vector capable of inducible expression where a *TipA* gene promoter that serves as a inducible promoter is substituted for at least a promoter in the vector of any one of [22] to [25];

[30] The bacterium belonging to the genus *Rhodococcus* of any one [27] to [29], wherein one of the at least two types of plasmid vectors is a vector of any one of [4], [5], [15] to [19], [20], and [21] and another is a vector selected from the group consisting of pTip-NH1 having a nucleotide sequence represented by SEQ ID NO: 49, pTip-NH2 having a nucleotide sequence represented by SEQ ID NO: 50, pTip-CH1 having a nucleotide sequence represented by SEQ ID NO: 51, pTip-CH2 having a nucleotide sequence represented by SEQ ID NO: 52, pTip-LNH1 having a nucleotide sequence represented by SEQ ID NO: 53, pTip-LNH2 having a nucleotide sequence represented by SEQ ID NO: 54, pTip-LCH1 having a nucleotide sequence represented by SEQ ID NO: 55, pTip-LCH2 having a nucleotide sequence represented by SEQ ID NO: 56, pTip-QT1 having a nucleotide sequence represented by SEQ ID NO: 91, pTip-QT2 having a nucleotide sequence represented by SEQ ID NO: 92, pTip-QC1 having a nucleotide sequence represented by SEQ ID NO: 95, pTip-QC2 having a nucleotide sequence represented by SEQ ID NO: 96, pTip-CH1.1, pTip-CH2.1, pTip-LCH1.1, pTip-LCH2.1, a vector of any one of [22] to [25], or a vector capable of inducible expression where a *TipA* gene promoter that serves as a inducible promoter is substituted for at least a promoter in the vector of any one of [22] to [25];

[31] The bacterium belonging to the genus *Rhodococcus* of any one of [26] to [30], wherein the DNA sequence essential for the rolling circle mode of replication is a DNA sequence at positions 3845 to 5849 in a nucleotide sequence represented by SEQ ID NO: 90 and the DNA sequence necessary for the autonomous replication of a plasmid derived from pRE2895 is a DNA sequence of a 1.9-kb region containing *RepA* and *RepB* genes;

[32] A method of producing foreign proteins by transforming a bacterium belonging to the genus *Rhodococcus* with at least two types of expression plasmid vectors, culturing the bacterium, and coexpressing genes encoding foreign proteins that are respectively contained in

the expression vectors under a temperature condition of 4°C to 35°C, the bacterium belonging to the genus *Rhodococcus* comprising at least the two types of expression plasmid vectors derived from the bacterium belonging to the genus *Rhodococcus* that do not cause plasmid incompatibility with each other and comprise the genes encoding foreign protein, the at least two types of vectors each having a DNA sequence that is derived from the bacterium belonging to the genus *Rhodococcus* and has the rolling circle mode of replication and a DNA sequence necessary for the autonomous replication of a plasmid derived from pRE2895 as DNA sequences necessary for the autonomous replication of a plasmid;

[33] The method of [32], wherein all of the two types of plasmid vectors respectively comprise a promoter sequence for foreign protein production, a ribosome-binding site sequence located downstream of the promoter sequence, and a multiple-cloning site sequence capable of incorporating a foreign gene therein, located downstream of the ribosome-binding site sequence;

[34] The method of any one of [32] to [33], wherein one of the at least two types of plasmid vectors is a vector of any one of [4], [5], [15] to [19], [20], and [21] and another is a vector selected from the group consisting of pTip-NH1 having a nucleotide sequence represented by SEQ ID NO: 49, pTip-NH2 having a nucleotide sequence represented by SEQ ID NO: 50, pTip-CH1 having a nucleotide sequence represented by SEQ ID NO: 51, pTip-CH2 having a nucleotide sequence represented by SEQ ID NO: 52, pTip-LNH1 having a nucleotide sequence represented by SEQ ID NO: 53, pTip-LNH2 having a nucleotide sequence represented by SEQ ID NO: 54, pTip-LCH1 having a nucleotide sequence represented by SEQ ID NO: 55, pTip-LCH2 having a nucleotide sequence represented by SEQ ID NO: 56, pTip-QT1 having a nucleotide sequence represented by SEQ ID NO: 91, pTip-QT2 having a nucleotide sequence represented by SEQ ID NO: 92, pTip-QC1 having a nucleotide sequence represented by SEQ ID NO: 95, pTip-QC2 having a nucleotide sequence represented by SEQ ID NO: 96, pTip-CH1.1, pTip-CH2.1, pTip-LCH1.1, pTip-LCH2.1, a vector of any one of [22] to [25], or a vector capable of inducible expression where a *TipA* gene promoter that serves as a inducible promoter is substituted for at least a promoter in the vector of any one of [22] to [25]; and

[35] The method of any one of [32] to [34], wherein a nucleotide sequence of the DNA essential for the rolling circle mode of replication is a nucleotide sequence at positions 3845 to 5849 in a nucleotide sequence represented by SEQ ID NO: 90 and the DNA sequence necessary for the autonomous replication of a plasmid derived from pRE2895 is DNA composed of a 1.9-kb region containing *RepA* and *RepB* genes.

Hereinafter, the present invention will be described in detail.

The present invention encompasses a circular plasmid capable of replicating in the rolling-circle mode, isolated from a bacterium belonging to the genus *Rhodococcus*, and an expression vector constructed from the circular plasmid. The rolling circle mode of replication refers to a mode of replication of double-stranded circular DNA, in which a particular site on a particular DNA strand is nicked by the action of specific endonuclease and DNA synthesis starts from the 3'-OH end of the nicked site and takes a round of the circle with an unnicked circular DNA strand as a template. Such a mode of replication requires a DNA region necessary for the rolling circle mode of replication. Examples of the DNA region include a *Rep* gene. The rolling circle mode of replication further requires a double-stranded origin (DSO) and a single-stranded origin (SSO). Thus, the circular plasmid capable of replicating in the rolling-circle mode and the expression vector constructed from the circular plasmid of the present invention are a plasmid and an expression vector comprising a DNA region necessary for the rolling circle mode of replication, that is, a *Rep* gene, a double-stranded origin (DSO), and a single-stranded origin (SSO). Such a plasmid can be isolated from a bacterium belonging to the genus *Rhodococcus*. Examples of the plasmid include pRE8424 isolated from a *Rhodococcus erythropolis* strain DSM8424, and the full-length sequence of the pRE8424 is shown in SEQ ID NO: 90. In SEQ ID NO: 90, a region at positions 3845 to 5849 represents the DNA region necessary for the rolling circle mode of replication, that is, the DNA of the *Rep* gene, the double-stranded origin (DSO), and the single-stranded origin (SSO).

The present invention also encompasses a plasmid capable of replicating in the rolling-circle mode, which is composed of DNA that hybridizes under stringent conditions to DNA complementary to DNA constituting a plasmid represented by SEQ ID NO: 90. The

stringent conditions used here refer to, for example, such conditions that a sodium concentration is 500 to 1000 mM, preferably 700 mM, and a temperature is 50 to 70°C, preferably 65°C. Such a plasmid is a plasmid whose full-length nucleotide sequence has 90% or more homology, preferably 95% or more homology, more preferably 98% or more homology to a nucleotide sequence represented by SEQ ID NO: 90 in calculation using BLAST or the like (e.g., using a default, i.e., a initialized parameter).

The present invention further encompasses an expression vector comprising a *Rep* gene, a double-stranded origin (DSO), and a single-stranded origin (SSO) as a DNA region necessary for the rolling circle mode of replication obtained from the plasmid and further comprising a promoter sequence, a ribosome-binding site sequence located downstream of the promoter sequence, and a multiple-cloning site sequence capable of incorporating a foreign gene therein, located downstream of the ribosome-binding site sequence. The expression vector may further contain a foreign gene and a transcription termination sequence. The DNA sequence having promoter activity, the foreign gene, and the transcription termination sequence constitute an expression cassette. The promoter sequence used here includes a promoter capable of inducer (such as a drug)-inducible expression of a foreign gene introduced downstream thereof and a promoter capable of inducer-independent and constitutive expression of a foreign gene. Examples of the former promoter capable of inducible expression of a foreign gene include a *TipA* gene promoter that inducibly expresses a foreign gene located downstream thereof in the presence of thiostrepton. The vector of the present invention may comprise a *TipA* gene encoding a TipA protein and an appropriate promoter inducing the expression of the *TipA* gene, such as a *ThcA* gene promoter. The *TipA* gene and the promoter for the expression of the *TipA* gene constitute an inducer cassette. When a host cell is a bacterium belonging to the genus *Rhodococcus*, a thiostrepton resistance gene or the like that imparts resistance to thiostrepton is incorporated into the vector because the bacterium is sensitive to thiostrepton. In addition, the *TipA* gene promoter may be any of those obtained by modifying the sequence of the *TipA* gene promoter, such as a *TipA-LG10* promoter. The sequence of the *TipA* gene promoter is shown in Figure 12.

Examples of the latter promoter capable of constitutive expression of a foreign gene include a modified promoter of the *TipA* gene promoter. Examples of such a modified *TipA* gene promoter include a promoter where a mutation is introduced into a -10 region sequence of the *TipA* gene promoter, and specifically include a promoter where the mutation in the -10 region sequence is a mutation of a CAGCGT sequence to a TATAAT sequence. Such a promoter can be exemplified by a promoter contained in a sequence shown in Figure 19.

A polynucleotide consisting of DNA that hybridizes under stringent conditions to DNA complementary to DNA having a promoter sequence shown in Figure 12 or DNA having a promoter sequence contained in a sequence shown in Figure 19, and having activity equivalent to the activity of each of the promoter sequence can also be used as the promoter. The stringent conditions used here refer to, for example, such conditions that a sodium concentration is 500 to 1000 mM, preferably 700 mM, and a temperature is 50 to 70°C, preferably 65°C. Such a polynucleotide is a promoter whose full-length nucleotide sequence has 90% or more homology, preferably 95% or more homology, more preferably 98% or more homology to the nucleotide sequences of the above-described promoters in calculation using BLAST or the like (e.g., using a default, i.e., a initialized parameter).

The present invention further includes a vector composed of the above-described vector and further comprising a DNA region necessary for the autonomous replication of a plasmid for *Escherichia coli* and a selection marker for the transformant of *Escherichia coli*. Such a vector can be used as a shuttle vector between a bacterium belonging to the genus *Rhodococcus* and *Escherichia coli*. In this case, the vector can be used as a constitutive expression vector in *Escherichia coli*. Any of those known in the art such as *Col*E1 and *Col*E2 sequences can be used as the DNA region necessary for the autonomous replication of a plasmid for *Escherichia coli*, and any of those known in the art such as an ampicillin resistance gene can be used as the selection marker for the transformant of *Escherichia coli*. These can be obtained from a cloning vector for *Escherichia coli* known in the art.

An expression vector for *Rhodococcus* comprising a *TipA* gene promoter, and a DNA region necessary for the rolling circle mode of replication, that is, a double-stranded origin (DSO) and a single-stranded origin (SSO), and further comprising a ribosome-binding site

sequence located downstream of the promoter sequence, a multiple-cloning site sequence capable of incorporating a foreign gene therein, located down stream of the ribosome-binding site sequence, and a DNA region necessary for the autonomous replication of a plasmid for *Escherichia coli* can be exemplified by pTip-RT1 having a nucleotide sequence represented by SEQ ID NO: 93, pTip-RT2 having a nucleotide sequence represented by SEQ ID NO: 94, pTip-RC1 having a nucleotide sequence represented by SEQ ID NO: 97, and pTip-RC2 having a nucleotide sequence represented by SEQ ID NO: 98. A vector having, instead of the *TipA* gene promoter, a promoter where a mutation in the -10 region sequence of the *TipA* gene promoter is a mutation of a CAGCGT sequence to a TATAAT sequence can be exemplified by pNit-RT1 having a nucleotide sequence represented by SEQ ID NO: 101, pNit-RT2 having a nucleotide sequence represented by SEQ ID NO: 102, pNit-RC1 having a nucleotide sequence represented by SEQ ID NO: 105, and pNit-RC2 having a nucleotide sequence represented by SEQ ID NO: 106. The present invention also encompasses a vector composed of DNA that hybridizes under stringent conditions to DNA complementary to DNA composed of any of the nucleotide sequences represented by these SEQ ID NOs and capable of expressing a foreign gene in a host microorganism. The stringent conditions used here refer to, for example, such conditions that a sodium concentration is 500 to 1000 mM, preferably 700 mM, and a temperature is 50 to 70°C, preferably 65°C. Such a vector is a vector whose full-length nucleotide sequence has 90% or more homology, preferably 95% or more homology, more preferably 98% or more homology to the nucleotide sequences represented by SEQ ID NOs of the above-described vectors in calculation using BLAST or the like (e.g., using a default, i.e., a initialized parameter). The same goes for vectors represented below by SEQ ID NOs.

The present invention also encompasses an expression vector comprising an alternative DNA region necessary for autonomous replication, other than the above-described DNA region (*Rep* gene, DSO, and SSO) necessary for the rolling circle mode of replication. Expression vectors having different DNA regions necessary for replication can be introduced simultaneously into a single host and maintained stably. Examples of the alternative DNA region necessary for autonomous replication include a *RepA* gene and a *RepB* gene. A DNA

region containing the *RepA* gene and the *RepB* gene can be isolated from an endogenous plasmid pRE2895 separated from a bacterium belonging to the genus *Rhodococcus*, for example, a *R. erythropolis* strain JCM2895. A 1.9-kb region containing the *RepA* gene and the *RepB* gene is a region at positions 6233 to 8166 in SEQ ID NO: 49, of which *RepA* ORF is located at positions 6756 to 7652 and *RepB* ORF is located at positions 7652 to 7936. The DNA region containing the *RepA* gene and the *RepB* gene can be obtained by reference to the restriction map (Figure 1) of a vector pHN129 described in Reference Example below. DNA that hybridizes under stringent conditions to DNA complementary to DNA consisting of a nucleotide sequence represented by positions 6233 to 8166 in SEQ ID NO: 49 and imparts autonomous replication ability to a vector can also be used as the 1.9-kb region containing the *RepA* gene and the *RepB* gene of the present invention. The stringent conditions used here refer to, for example, such conditions that a sodium concentration is 500 to 1000 mM, preferably 700 mM, and a temperature is 50 to 70°C, preferably 65°C. Such DNA is DNA whose full-length nucleotide sequence has 90% or more homology, preferably 95% or more homology, more preferably 98% or more homology to the nucleotide sequence represented by positions 6233 to 8166 in SEQ ID NO: 49 in calculation using BLAST or the like (e.g., using a default, i.e., a initialized parameter). An expression vector comprising this DNA region necessary for autonomous replication, a promoter having a -10 region sequence of a *TipA* gene promoter mutated from a CAGCGT sequence to a TATAAT sequence, a ribosome-binding site sequence located downstream of the promoter, and a multiple-cloning site sequence capable of incorporating a foreign gene therein, located downstream of the ribosome-binding site sequence allows the inducer-independent and constitutive expression of the foreign gene incorporated in the multiple-cloning site. Examples of such an expression vector include a constitutive expression vector for a bacterium belonging to the genus *Rhodococcus* selected from the group consisting of pNit-QT1 having a nucleotide sequence represented by SEQ ID NO: 99, pNit-QT2 having a nucleotide sequence represented by SEQ ID NO: 100, pNit-QC1 having a nucleotide sequence represented by SEQ ID NO: 103, and pNit-QC2 having a nucleotide sequence represented by SEQ ID NO: 104, and further include pTip-NH1 having a nucleotide sequence represented by SEQ ID NO: 49, pTip-NH2 having a nucleotide sequence

represented by SEQ ID NO: 50, pTip-CH1 having a nucleotide sequence represented by SEQ ID NO: 51, pTip-CH2 having a nucleotide sequence represented by SEQ ID NO: 52, pTip-LNH1 having a nucleotide sequence represented by SEQ ID NO: 53, pTip-LNH2 having a nucleotide sequence represented by SEQ ID NO: 54, pTip-LCH1 having a nucleotide sequence represented by SEQ ID NO: 55, pTip-LCH2 having a nucleotide sequence represented by SEQ ID NO: 56, and a vector where the promoter having a -10 region sequence of the *TipA* gene promoter mutated from a CAGCGT sequence to a TATAAT sequence is substituted for the inducible promoter of pTip-CH1.1, pTip-CH2.1, pTip-LCH1.1, and pTip-LCH2.1, all of which allow the inducible expression of an introduced foreign gene in the presence of thiostrepton. It is noted that the inducible expression vector needs to further contain an inducer cassette containing a *TipA* gene or a mutant thereof and a promoter for the expression of the *TipA* gene as well as a thiostrepton resistance gene.

A foreign gene can be expressed by incorporating the foreign gene into the above-described expression vector of the present invention, introducing the expression vector into a host microorganism, and culturing the host microorganism. The incorporation of the foreign gene into the expression vector can be performed by a genetic engineering approach known in the art, and the introduction of the expression vector into the host microorganism can also be performed by an approach known in the art. In addition, the culture of the host microorganism may be performed under appropriate conditions using a medium suitable for each microorganism. Examples of a host organism into which the vector is incorporated include bacteria belonging to the genus *Rhodococcus* and *Escherichia coli*. The foreign gene used here refers to a gene encoding a target protein to be expressed using the vector of the present invention and encoding a protein derived from an organism other than a host cell. The protein to be expressed and produced using the vector of the present invention is not limited, and any protein can be the target protein. When a host organism into which the expression vector of the present invention is introduced is a microorganism capable of proliferation at low temperatures, for example, a bacterium belonging to the genus *Rhodococcus* such as *R. erythropolis*, *R. fascians*, and *R. opacus*, it is possible to express and produce a protein whose expression is difficult or impossible under a typical temperature

condition suitable for the proliferation of a microorganism, that is, at moderate and high temperatures exceeding approximately 15°C. Examples of such a protein include: a protein that can not be expressed at temperatures falling within the optimum growth temperature range of a host cell but can be expressed at temperatures lower than temperatures falling within the preferred growth temperature range of the same or a different type of host microorganism cell; a protein that is lethal to a host microorganism cell when expressed at temperatures falling within the preferred growth temperature range of the host microorganism cell but is not lethal its host cell at temperatures lower than temperatures falling within the preferred growth temperature ranges of the same or the different type of host cell; a protein that inhibits the proliferation of a host cell when expressed at temperatures falling within the preferred growth temperature range of the host cell but does not inhibit the proliferation of its host cell at temperatures lower than temperatures falling within the preferred growth temperature ranges of the same or the different type of host cell; a protein that forms the agglutination of inactive proteins called an inclusion body when expressed at temperatures falling within the preferred growth temperature range of a host cell but becomes a soluble protein with activity when expressed in its host cell at temperatures lower than temperatures falling within the preferred growth temperature ranges of the same or the different type of host cell; and a protein derived from a psychrophile with a preferred growth temperature range not higher than 20°C, from a poikilotherm surviving under low temperature atmosphere, and from a plant surviving under low temperature atmosphere.

When a promoter contained in the expression vector is an inducible promoter, the expression and production of a foreign gene can be induced by adding an inducer to the culture medium of a host microorganism. Examples of the inducible promoter contained in the expression vector of the present invention include a *TipA* gene promoter. When the gene promoter is contained, the expression and production of a foreign gene is induced by the addition of thiostrepton. In this case, the thiostrepton may be added at a final concentration of 0.1 μg/ml or more, preferably 1 μg/ml or more. However, the addition of thiostrepton at a final concentration of 10 μg/ml or more deteriorates the growth of a host. When the

expression vector of the present invention contains a constitutive promoter, a foreign gene is expressed and produced without the addition of an inducer.

Of the expression vectors of the present invention, the expression vectors having different DNA regions necessary for autonomous replication from each other can be cotransformed simultaneously into a single microorganism cell and thereby maintained stably in the cell to simultaneously express and produce foreign genes respectively contained in the vectors. In this case, the foreign genes respectively contained in the vectors may be those encoding the same or different proteins. For example, the subunits of a protein consisting of two subunits are respectively incorporated into separate expression vectors having different DNA regions necessary for autonomous replication from each other, and the expression vectors are introduced into an identical microorganism cell. As a result, the subunits are simultaneously expressed in the single cell and associated with each other to produce a complete protein. In this case, the expression vectors may be used in any combination of those capable of constitutive expression of a foreign gene and those capable of inducible expression of a foreign gene. Expression vectors capable of inducible expression of foreign genes are used as all of several expression vectors having different DNA regions necessary for autonomous replication, and the expression of the foreign genes is induced by an expression inducer, thereby allowing the simultaneous expression and production of two or more types of foreign proteins.

In addition, two types of proteins can also be expressed simultaneously in *Escherichia coli* by selecting different replications origins for *Escherichia coli* for the expression vectors of the present invention.

The present specification encompasses the contents described in the specification and/or drawings of Japanese Patent Application No. 2003-116280 that serves as a basis for the priority of the present application.

Brief Description of the Drawings

Figure 1 is a diagram for illustrating the construction of a plasmid pHN136 that serves as the backbone of inducible expression vectors. In the drawing, the locations of restriction

enzyme recognition sites and structural genes are shown. A numeric represents a base pair (kilobase pair: kb);

Figure 2 is a diagram for illustrating the construction of a plasmid pHN143 having a thiostrepton resistance gene. In the drawing, the locations of restriction enzyme recognition sites and structural genes are shown. A numeric represents a base pair (kilobase pair: kb). CIAP means calf intestine alkaline phosphatase, and Blu. means a blunt end;

Figure 3 is a diagram for illustrating the construction of a plasmid pHN62 having an inducer cassette. In the drawing, the locations of restriction enzyme recognition sites and structural genes are shown. A numeric represents a base pair (kilobase pair: kb). Blu. means a blunt end;

Figure 4 is a diagram for illustrating the construction of a plasmid pHN153 having an expression cassette. In the drawing, the locations of restriction enzyme recognition sites and structural genes are shown. A numeric represents a base pair (kilobase pair: kb). CIAP means calf intestine alkaline phosphatase, and Blu. means a blunt end;

Figure 5 is a diagram for illustrating the construction of a plasmid pHN169 having a tetracycline resistance gene. In the drawing, the locations of restriction enzyme recognition sites and structural genes are shown. A numeric represents a base pair (kilobase pair: kb). CIAP means calf intestine alkaline phosphatase, and Blu. means a blunt end;

Figure 6 is a diagram for illustrating the construction of inducible expression vector plasmids pHN170 and pHN171 having a *PIP* gene as a reporter gene. In the drawing, the locations of restriction enzyme recognition sites and structural genes are shown. A numeric represents a base pair (kilobase pair: kb). CIAP means calf intestine alkaline phosphatase;

Figure 7 is a diagram for illustrating the construction of inducible expression vector plasmids pTip-NH1, pTip-CH1, pTip-LNH1, and pTip-LCH1 having a multiple-cloning site. In the drawing, the locations of restriction enzyme recognition sites and structural genes are shown. A numeric represents a base pair (kilobase pair: kb);

Figure 8 is a diagram for illustrating the construction of inducible expression vector plasmids pTip-NH2, pTip-CH2, pTip-LNH2, and pTip-LCH2 having a multiple-cloning site.

In the drawing, the locations of restriction enzyme recognition sites and structural genes are shown. A numeric represents a base pair (kilobase pair: kb);

Figure 9a is a diagram showing the maps of the plasmids pTip-NH1, pTip-CH1, pTip-LNH1, pTip-LNH1, pTip-NH2, pTip-CH2, pTip-LNH2, and pTip-LCH2. The function of each region and the maps of the plasmids are shown;

Figure 9b shows the DNA sequence of the pTip-NH1 or the pTip-LNH1 from a *TipA* gene promoter sequence or a *TipA-LG10* promoter sequence via a multiple-cloning site to a *ThcA* gene transcription termination sequence;

Figure 9c shows the DNA sequence of the pTip-CH1 or the pTip-LCH1 from a *TipA* gene promoter sequence or a *TipA-LG10* promoter sequence via a multiple-cloning site to a *ThcA* gene transcription termination sequence;

Figure 9d shows the DNA sequence of the pTip-NH2 or the pTip-LNH2 from a *TipA* gene promoter sequence or a *TipA-LG10* promoter sequence via a multiple-cloning site to a *ThcA* gene transcription termination sequence;

Figure 9e shows the DNA sequence of the pTip-CH2 or the pTip-LCH2 from a *TipA* gene promoter sequence or a *TipA-LG10* promoter sequence via a multiple-cloning site to a *ThcA* gene transcription termination sequence;

Figure 10 is a diagram showing the maps of pTip-CH1.1, pTip-LCH1.1, pTip-CH2.1, and pTip-LCH2.1;

Figure 11 is a diagram for illustrating the construction of control plasmids pHN172 and pHN173 for the activity measurement of PIP. In the drawing, the locations of restriction enzyme recognition sites and structural genes are shown. A numeric represents a base pair (kilobase pair: kb). CIAP means calf intestine alkaline phosphatase. The pHN170 has both of an "expression cassette" and an "inducer cassette", while the pHN173 has only the "expression cassette" and the pHN172 lacks both cassettes;

Figure 12 is a diagram showing a *TipA* gene promoter sequence;

Figure 13 is a diagram showing the modification of a *TipA* gene promoter to a *TipA-LG10* promoter;

Figure 14 is a diagram showing the map of pRE8424. In the drawing, main restriction enzyme recognition sites are shown, and an open reading frame (ORF) is indicated by an arrow. The locations of DSO and SSO are indicated by a box;

Figure 15 is diagram showing the amino acid sequences of five motifs (Motif IV, Motif I, Motif II, Motif III, and C-terminal motif) that are conserved in Rep proteins among pRE8424, pAP1, pBL1, pJV1, pIJ101, and pSN22. A tyrosine residue allegedly important for the function of the Rep protein is boxed;

Figure 16 is a diagram showing an especially conserved DNA sequence, of sequences likely to be the DSOs of the pRE8424, the pAP1, the pBL1, the pJV1, the pIJ101, and the pSN22;

Figure 17 is a diagram showing the SSO of the pRE8424, that is, a sequence of nucleotide Nos. 5268 to 5538 in SEQ ID NO: 90 in the sequence listing, and a possible secondary structure;

Figure 18-1 is a diagram showing the map of a pTip vector;

Figure 18-2 is a diagram showing the map of a pNit vector;

Figure 19 is a diagram showing the DNA sequence of *TipA-LG10p*-MCS-*ALDHt* or *Nit-LG10*-MCS-*ALDHt*. A wild-type -10 region sequence of a *TipA* gene promoter is CAGCGT, and a -10 region sequence of a *Nit* promoter is TATAAT. These sequences are boxed, respectively;

Figure 20 is a diagram showing a result of measuring PIP for peptidase activity, after the transformation of a *R. erythropolis* strain JCM3201 with pHN380, pHN410, pHN381, pHN387, and pHN389; and

Figure 21 is a photograph showing a result obtained by the following procedures: *PIP* and *GFP* genes are incorporated into two vectors that do not cause incompatibility with each other, PIP and GFP expressed in a single *R. erythropolis* strain JCM3201 cell are purified and analyzed by SDS polyacrylamide electrophoresis, followed by the staining of the gels with Coomassie Brilliant Green G-250.

Best Mode for Carrying Out the Invention

Hereinafter, the present invention will be described more fully with reference to Examples. However, the technical scope of the present invention is not intended to be limited to these Examples.

[Reference Example 1]

(1) Separation of a *Rhodococcus erythropolis*-derived plasmid capable of autonomous replication in bacterium belonging to the genus *Rhodococcus* and determination of its partial DNA sequence

For constructing a shuttle vector between *Rhodococcus erythropolis* and *Escherichia coli*, a small endogenous plasmid present in a bacterium belonging to the genus *Rhodococcus* was initially searched. As a result, its presence was confirmed in a *Rhodococcus erythropolis* strain JCM2895. This plasmid was designated as pRE2895. Hereinafter, the separation of the plasmid and the determination of its DNA sequence will specifically be described.

The QIAprep Spin Miniprep Kit (manufactured by QIAGEN) was used to purify pRE2895 from the cells of a *Rhodococcus erythropolis* strain JCM2895 cultured at 30°C for 30 hours in 5 ml of a LB medium (1% Difco Bacto Tryptone, 0.5% Difco Yeast Extract, and 1.0 % sodium chloride). These procedures were conducted according to the instruction except that the bacterial cells were supplemented with 5 μl of lysozyme (100 mg/ml) and incubated at 37°C for 30 minutes before the addition of 250 μl of Buffer P2 following suspension in 250 μl of Buffer P1.

When the above-described DNA sample was treated with a restriction enzyme *Eco*RI and subjected to 1.0% agarose gel electrophoresis (100 V, 30 minutes), the presence of one DNA fragment of approximately 5.4 kb was confirmed.

This DNA fragment of approximately 5.4 kb was cut out of the gel and purified using the QIAquick Gel Extraction Kit (manufactured by QIAGEN) according to the instruction. The obtained *Eco*RI fragment was subcloned into the *Eco*RI site of a plasmid pBluescript II SK(+) (manufactured by STRATAGENE) according to an ordinary method (Sambrook et al., Molecular Cloning: a laboratory manual, 2nd edition [1989], Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.). This plasmid was designated as pHN79.

The nucleotide sequence of the pHN79 was determined by approximately 400 bases at a time using both reverse and M13-20 primers (both manufactured by STRATAGENE) and using a DNA sequencer ABI PRISM(R) 3100 Genetic Analyzer (manufactured by ABI) according to the instruction. As a result of homology search, 99.8% of the sequence of the *Rhodococcus erythropolis* strain JCM2895-derived DNA region subcloned into the pHN79 matched to that of pN30, a circular DNA having 5403 base pairs registered as Accession No. AF312210 in the GenBank.

The full nucleotide sequence of the separated pRE2895 was not determined. However, because the pRE2895 has high homology to the pN30 and its restriction enzyme cleavage map also matched to that predicted from the sequence of the pN30, this homology between the pRE2895 and the pN30 was estimated to extend throughout the plasmids. Moreover, the pN30 has high homology to an endogenous plasmid pAL5000 (Rauzer et al., Gene *71* 315-321 [1988]; and Stolt and Stoker, Microbiology *142* 2795-2802 [1996]) separated from a *Mycobacterium fortuitum* strain 002 and pFAJ2600 (De Mot et al., Microbiology *143* 3137-3147 [1997]) separated from a *Rhodococcus erythropolis* strain NI86/21, and they were considered to autonomously replicate in similar mechanisms. Because only a region containing a putative *RepA* gene, a putative *RepB* gene, and a putative replication origin is sufficient for the autonomous replication of the pAL5000 in each bacterium, the incorporation of only a similar region into an expression vector was also considered to be sufficient for the autonomous replication of the pRE2895 separated by the present inventors in a bacterium belonging to the genus *Rhodococcus*.

(2) Construction of a vector plasmid pHN136

To construct shuttle vector between *Escherichia coli* and *Rhodococcus erythropolis*, the following procedures were performed (Figure 1).

Synthetic oligodeoxyribonucleotide primers (hereinafter, abbreviated to primers) represented by SEQ ID NOs: 1 and 2 in the sequence listing were used to perform DNA amplification by a polymerase chain reaction method (hereinafter, abbreviated to PCR; Saiki et al., Science, *239* 487-491 [1988]) with a plasmid pBluescript II SK(-) (manufactured by STRATAGENE) as a template. An enzyme used for PCR is the Pfu turbo (manufactured by

STRATAGENE). As a result, a 2.0-kb amplified DNA containing an ampicillin resistance gene (indicated by Amp^r in the drawings) and a *Col*E1 sequence region necessary for autonomous replication in *Escherichia coli* was obtained. This DNA fragment was doubly digested with restriction enzymes *Sac*I and *Bsr*GI and subjected to 1.0% agarose gel electrophoresis (100 V, 30 minutes). The DNA fragment was cut out of the gel and purified using the QIAquick Gel Extraction Kit according to the instruction.

On the other hand, primers that amplify a region likely to be necessary for autonomous replication in a bacterium belonging to the genus *Rhodococcus* were designed on the basis of the sequence of the pN30 (the above (1)). The sequences of the primers are shown in SEQ ID NOs: 3 and 4 in the sequence listing. Both of the primers were used to perform amplification by PCR with the plasmid pHN79 as a template to yield a 1.9-kb amplified DNA. This DNA fragment was doubly digested with restriction enzymes *Bsr*GI and *Sac*I and subjected to 1.0% agarose gel electrophoresis (100 V, 30 minutes). The DNA fragment was cut out of the gel and purified in the same way as above.

These two purified DNA fragments were ligated to a plasmid using the DNA Ligation Kit Ver. 2 (manufactured by Takara Shuzo) according to the instruction. The resulting plasmid was designated as pHN129.

For eliminating restriction enzyme recognition sites *Bam*HI and *Sal*I present in the pHN129, the following procedures were performed. At first, primers represented by SEQ ID NOs: 5 and 6 in the sequence listing were used to perform amplification by PCR with the pHN129 as a template. A 0.5-kb DNA fragment obtained by doubly digesting this PCR fragment with *Bgl*II and *Pst*I was subcloned into the *Bam*HI and *Pst*I sites of the pHN129. Consequently, although the portion of the ligation between *Bgl*II and *Bam*HI was situated within the open reading frame (hereinafter, abbreviated to ORF) of the putative *Rep*A gene, the *Bam*HI recognition site was eliminated without any substitution in encoded amino acids. The *Sal*I recognition site had been located in close proximity to the *Bam*HI recognition site and however, was also eliminated simultaneously with the elimination of the *Bam*HI recognition site because the primer represented by SEQ ID NO: 5 was designed so that the

*Sal*I recognition site was eliminated without any substitution in encoded amino acids. This plasmid was designated as pHN135.

Next, for eliminating a restriction enzyme recognition site *Bgl*II present in the pHN135, the following procedures were performed. At first, primers represented by SEQ ID NOs: 5 and 6 in the sequence listing were used to perform amplification by PCR with the plasmid pHN135 as a template. A 0.5-kb DNA fragment obtained by doubly digesting this PCR fragment with *Pst*I and *Bam*HI was subcloned into the *Pst*I and *Bgl*II sites of the pHN135. Consequently, although the portion of the ligation between *Bam*HI and *Bgl*II was the ORF portion of the putative *Rep*B gene, the *Bgl*II recognition site was eliminated without any substitution in encoded amino acids. The resulting plasmid was designated as pHN136.

(3) Construction of a vector plasmid pHN143

Although an antibiotic thiostrepton is used for inducing protein expression, *Rhodococcus erythropolis* is sensitive to the antibiotic. Therefore, thiostrepton resistance must be imparted to *Rhodococcus erythropolis*. Thus, it has been decided that a thiostrepton resistance gene, a *tsr* gene, of *Streptomyces azureus* (Bibb et al., Mol. Gen. Genet. *199* 26-36 [1985]; indicated by Thio^r in the drawings) is incorporated into the shuttle vector. It has been already reported that this gene functions in *Rhodococcus erythropolis* and imparts thiostrepton resistance (Shao and Behki, Lett. Appl. Microbiol. *21* 261-266 [1995]). Hereinafter, the separation of the gene will specifically be described (Figure 2).

The genomic DNA of a *Streptomyces azureus* strain JCM4217 used as a PCR template was prepared as follows. The strain cultured at 30°C in 5 ml of a SB medium (1% Difco Bacto Tryptone, 0.5% Difco Yeast Extract, 0.5% sodium chloride, 0.1% glucose, 5 mM magnesium chloride, and 0.5% glycine) was suspended in 500 μl of a SET buffer (75 mM sodium chloride, 25 mM EDTA [pH 8.0], and 20 mM Tris-HCl [pH 7.5]). To this suspension, 5 μl of a lysozyme solution (100 mg/ml) was added and incubated at 37°C for 30 minutes. The resulting mixture solution was then supplemented with 14 μl of a protease K solution (20 mg/ml) and 60 μl of a sodium dodecyl sulfate solution (10%) and sufficiently mixed, followed by incubation at 55°C for 2 hours. The resulting mixture solution was then supplemented with 200 μl of a sodium chloride solution (5 M) and 500 μl of chloroform and

stirred by rotation at room temperature for 20 minutes. Following centrifugation, 700 μl of a supernatant was obtained. After this supernatant was subjected to isopropanol precipitation, the resulting DNA pellet was dried and dissolved in 50 μl of a TE solution (10 mM Tris-HCl [pH 8.0] and 1 mM EDTA [pH 8.0]).

Primers represented by SEQ ID NOs: 7 and 8 in the sequence listing were used to perform amplification by PCR using the genomic DNA of the *Streptomyces azureus* strain JCM4217 purified as described above as a template. As a result, a 1.1-kb amplified DNA containing the thiostrepton resistance gene was obtained. The ends of this DNA fragment were blunt ends because the Platinum Pfx DNA Polymerase (manufactured by Gibco BRL) was used in the PCR. This DNA fragment was purified, and its 5' ends were phosphorylated with T4 polynucleotide kinase according to an ordinary method (Sambrook et al., Molecular Cloning: a laboratory manual, 2nd edition [1989], Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.). Then, the DNA fragment was subcloned into the *Hinc*II site of a plasmid pGEM-3Zf(+) (manufactured by Promega) (subcloned in the orientation of *Hin*dIII recognition site-*tsr* gene ORF-*Eco*RI recognition site in the 5' to 3' direction of the DNA). This plasmid was designated as pHN137.

Next, for eliminating a restriction enzyme recognition site *Sal*I present in the pHN137, the following procedures were performed. At first, primers represented by SEQ ID NOs: 9 and 10 in the sequence listing were used to perform amplification by PCR with the plasmid pHN137 as a template. The Platinum Pfx DNA Polymerase was used in this PCR. A 0.6-kb DNA fragment obtained by digesting the ends on one side of this PCR fragment with *Hin*dIII was purified, and its 5' end on the blunt end side was phosphorylated with T4 polynucleotide kinase according to an ordinary method. On the other hand, primers represented by SEQ ID NOs: 11 and 12 in the sequence listing were used to perform amplification by PCR with the plasmid pHN137 as a template. The Platinum Pfx DNA Polymerase was used in this PCR. A 0.5-kb DNA fragment obtained by digesting the ends on one side of this PCR fragment with *Eco*RI was purified, and its 5' end on the blunt end side was phosphorylated with T4 polynucleotide kinase according to an ordinary method. These two PCR fragments were simultaneously subcloned into the *Hin*dIII and *Eco*RI sites of a

plasmid pGEM-3Zf(+). Consequently, although the portion of the ligation between blunt ends was the ORF portion of the *tsr* gene, the *Sal*I recognition site was eliminated without any substitution in the encoded amino acids. This plasmid was designated as pHN143.

(4) Construction of a vector plasmid pHN62

For thiostrepton-inducible expression, a TipA protein must be present in a bacterium belonging to the genus *Rhodococcus*. Therefore, a constitutive promoter was separated from *Rhodococcus erythropolis*, and a structural gene encoding the TipA protein was ligated downstream of the promoter (Figure 3). The promoter sequence of a *ThcA* gene (Nagy et al., J. Bacteriol. *177* 676-687 [1995]) encoding the aldehyde dehydrogenase-like protein of *Rhodococcus erythropolis* was used as the constitutively functioning promoter.

The genomic DNA of a *Streptomyces coelicolor* strain A3(2) used as a template was prepared and purified in the same procedures as those for the preparation of the genomic DNA from *Streptomyces azureus*. In addition, the genomic DNA of a *Rhodococcus erythropolis* strain JCM3201 was prepared and purified in the same procedures as those for the preparation of the genomic DNA from *Streptomyces azureus* except that the strain JCM3201 was cultured in 5 ml of a LB medium.

Primers represented by SEQ ID NOs: 13 and 14 in the sequence listing were used to perform amplification by PCR using the genomic DNA of the *Streptomyces coelicolor* strain A3(2) purified as described above as a template. The Platinum Pfx DNA Polymerase was used in this PCR. As a result, DNA (indicated by TipA in the drawings) containing the ORF of the *TipA* gene and a transcription termination sequence located downstream thereof was obtained.

A 0.9-kb DNA fragment obtained by digesting the ends on one side of this PCR fragment with *Bgl*II was purified, and its 5' end on the blunt end side was phosphorylated with T4 polynucleotide kinase according to an ordinary method. On the other hand, primers represented by SEQ ID NOs: 15 and 16 in the sequence listing were used to perform amplification by PCR using the genomic DNA of the *Rhodococcus erythropolis* strain JCM3201 purified as described above as a template. As a result, DNA containing the promoter sequence (indicated by ALDHp in the drawings) of the *ThcA* gene (Nagy et al., J.

Bacteriol. *177* 676-687 [1995]) encoding the aldehyde dehydrogenase-like protein was obtained. The Platinum Pfx DNA Polymerase was used in this PCR. A 0.2-kb DNA fragment obtained by digesting the ends on one side of this PCR fragment with *Xba*I was purified, and its 5' end on the blunt end side was phosphorylated with T4 polynucleotide kinase according to an ordinary method. These two PCR fragments were simultaneously subcloned into the *Xba*I and *Bam*HI sites of a plasmid pGEM-3Zf(+). Consequently, a plasmid containing the ORF of the *TipA* gene and the transcription termination sequence located immediately downstream of the promoter sequence of the *ThcA* gene was constructed and designated as pHN33.

Next, for eliminating two *Nco*I restriction enzyme recognition sites (hereinafter, indicated by *Nco*I(1) and *Nco*I(2)) present in the pHN33, the following procedures were performed.

At first, primers represented by SEQ ID NOs: 9 and 17 in the sequence listing were used to perform amplification by PCR with the plasmid pHN33 as a template. The Platinum Pfx DNA Polymerase was used in this PCR. A 0.5-kb DNA fragment obtained by digesting the ends on one side of this PCR fragment with *Xba*I was purified, and its 5' end on the blunt end side was phosphorylated with T4 polynucleotide kinase according to an ordinary method. On the other hand, primers represented by SEQ ID NOs: 18 and 12 in the sequence listing were used to perform amplification by PCR with the plasmid pHN33 as a template. The Platinum Pfx DNA Polymerase was used in this PCR. A 0.6-kb DNA fragment obtained by digesting the ends on one side of this PCR fragment with *Kpn*I was purified, and its 5' end on the blunt end side was phosphorylated with T4 polynucleotide kinase according to an ordinary method. These two PCR fragments were simultaneously subcloned into the *Xba*I and *Kpn*I sites of a plasmid pGEM-3Zf(+). Consequently, although the portion of the ligation between blunt ends was the ORF portion of the *TipA* gene, the *Nco*I(1) recognition site was eliminated without any substitution in encoded amino acids. This plasmid was designated as pHN50.

Next, for eliminating the restriction enzyme recognition site *Nco*I(2) present in the pHN33, the following procedures were performed. At first, primers represented by SEQ ID NOs: 9 and 19 in the sequence listing were used to perform amplification by PCR with the

plasmid pHN33 as a template. The Platinum Pfx DNA Polymerase was used in this PCR. A 0.8-kb DNA fragment obtained by digesting the ends on one side of this PCR fragment with *Xba*I was purified, and its 5' end on the blunt end side was phosphorylated with T4 polynucleotide kinase according to an ordinary method. On the other hand, primers represented by SEQ ID NOs: 20 and 12 in the sequence listing were used to perform amplification by PCR with the plasmid pHN33 as a template. The Platinum Pfx DNA Polymerase was used in this PCR. A 0.3-kb DNA fragment obtained by digesting the ends on one side of this PCR fragment with *Kpn*I was purified, and its 5' end on the blunt end side was phosphorylated with T4 polynucleotide kinase according to an ordinary method. These two PCR fragments were simultaneously subcloned into the *Xba*I and *Kpn*I sites of a plasmid pGEM-3Zf(+). Consequently, although the portion of the ligation between blunt ends was the ORF portion of the *TipA* gene, the *Nco*I(2) recognition site was eliminated without any substitution in encoded amino acids. This plasmid was designated as pHN51.

In the last place, the following procedures were performed. A 0.7-kb DNA fragment obtained by doubly digesting the pHN50 with *Xba*I and *Sac*I and a 0.4-kb fragment obtained by doubly digesting the pHN51 with *Sac*I and *Kpn*I were simultaneously subcloned into the *Xba*I and *Kpn*I sites of a plasmid pGEM-3Zf(+). Consequently, a plasmid having the *TipA* gene but lacking both *Nco*I(1) and *Nco*I(2) restriction sites was obtained and designated as pHN62.

(5) Construction of a vector plasmid pHN153

For confirming whether a protein of interest can be inducibly expressed, the ORF (indicated by PIP ORF in the drawings) of a gene encoding *Thermoplasma acidophilum*-derived proline iminopeptidase (Tamura et al., FEBS Lett. *398* 101-105 [1996]; hereinafter, abbreviated to PIP) was ligated, as a reporter gene, downstream of the *TipA* gene promoter, and a transcription termination sequence was further ligated downstream of the ORF for suppressing transcriptional readthrough. Hereinafter, the procedures will specifically be described (Figure 4).

Primers represented by SEQ ID NOs: 21 and 22 in the sequence listing were used to perform amplification by PCR using the genomic DNA of the *Streptomyces coelicolor* strain

A3(2) purified in the above (4) as a template. As a result, a 0.2-kb amplified DNA containing the *TipA* gene promoter sequence (indicated by TipAp in the drawings) was obtained. The Platinum Pfx DNA Polymerase was used in this PCR. This DNA fragment was purified, and its 5' ends were phosphorylated with T4 polynucleotide kinase according to an ordinary method. Then, the DNA fragment was subcloned into the *Sma*I site of a plasmid pBluescript II SK(+) (subcloned in the orientation of *Kpn*I recognition site-*TipA* gene promoter sequence-*Sac*I recognition site in the 5' to 3' direction of the DNA). This plasmid was designated as pHN150u.

Next, primers represented by SEQ ID NOs: 23 and 24 in the sequence listing were used to perform amplification by PCR with a plasmid pRSET-PIP (Tamura et al., FEBS Lett. *398* 101-105 [1996]; hereinafter, abbreviated to PIP) as a template. The primer represented by SEQ ID NO: 24 in the sequence listing is designed so that 6xHis tag is attached to the C terminus of a PIP protein in order to eliminate the termination codon of the *PIP* gene and facilitate protein purification. The 6xHis tag is a consecutive sequence consisting of six consecutive histidine residues, and a protein fused with this tag exhibits high affinity for a nickel ion or the like. Thus, the protein is readily purified by metal chelate chromatography that employs the nickel ion or the like (Crowe et al., Methods Mol. Biol. *31* 371-387 [1994]). This 0.9-kb DNA fragment containing the *PIP* gene was doubly digested with restriction enzymes *Nco*I and *Spe*I and subcloned into the *Nco*I and *Spe*I sites of the pHN150u. Consequently, a plasmid containing the ORF of the *PIP* gene located immediately downstream of the *TipA* gene promoter sequence was constructed and designated as pHN151u.

Next, primers represented by SEQ ID NOs: 25 and 26 in the sequence listing were used to perform amplification by PCR using the genomic DNA of the *Rhodococcus erythropolis* strain JCM3201 purified in the above (4) as a template. As a result, DNA containing the transcription termination sequence (Nagy et al., J. Bacteriol. *177* 676-687 [1995], indicated by ALDHt in the drawings) of the *ThcA* gene was obtained. This 0.2-kb DNA fragment was doubly digested with restriction enzymes *Spe*I and *Xba*I and subcloned into the *Spe*I and *Xba*I sites of the pHN151u. Consequently, a plasmid containing the ORF of the *PIP* gene located immediately downstream of the *TipA* gene promoter sequence and the transcription

termination sequence of the *ThcA* gene located immediately downstream of the ORF was constructed and designated as pHN153.

(6) Construction of a vector plasmid pHN169

The transformation of *Rhodococcus erythropolis* with a plasmid requires an appropriate transformation marker. Thus, it has been decided to use a drug resistance gene ligated downstream of a strong promoter that functions in a bacterium belonging to the genus *Rhodococcus*. It has been decided to use, as the promoter, the promoter of a *Streptomyces* bacterium-derived *Tuf1* gene encoding an elongation factor Tu. This is because this promoter has been reported to strongly direct the transcription of a gene located downstream thereof (Wezel et al., Biochim. Biophys. Acta *1219* 543-547 [1994]). Besides, a tetracycline resistance gene easily available was used as the drug resistance gene. Hereinafter, the procedures will specifically be described (Figure 5).

Primers represented by SEQ ID NOs: 27 and 28 in the sequence listing were used to perform amplification by PCR using the genomic DNA of the *Streptomyces coelicolor* strain A3(2) purified in the above (4) as a template. As a result, a 0.2-kb amplified DNA containing the *Tuf1* gene promoter sequence (indicated by Tuf1p in the drawings) was obtained. The Platinum Pfx DNA Polymerase was used in this PCR. This DNA fragment was purified, and its 5' ends were phosphorylated with T4 polynucleotide kinase according to an ordinary method. Then, the DNA fragment was subcloned into the *Hinc*II site of a plasmid pBluescript II SK(+) (subcloned in the orientation of *Kpn*I recognition site-*Tuf1* gene promoter sequence-*Eco*RI recognition site in the 5' to 3' direction of the DNA). This plasmid was designated as pHN158.

Next, primers represented by SEQ ID NOs: 29 and 30 in the sequence listing were used to perform amplification by PCR with a plasmid pACYC184 (Rose, Nucleic Acids Res. *16* 355 [1988]) as a template. As a result, DNA containing the tetracycline resistance gene (indicated by Tet^r in the drawings) was obtained. This 1.3-kb DNA fragment was doubly digested with restriction enzymes *Xho*I and *Spe*I and subcloned into the *Sal*I and *Spe*I sites of the pHN158. Consequently, a plasmid containing the tetracycline resistance gene located

immediately downstream of the *Tuf1* gene promoter sequence was constructed and designated as pHN159.

Next, for eliminating a restriction enzyme recognition site *Bam*HI present in the pHN159, the following procedures were performed. At first, primers represented by SEQ ID NOs: 31 and 32 in the sequence listing were used to perform amplification by PCR with the plasmid pHN159 as a template. This DNA fragment had blunt ends because the Pfu turbo DNA Polymerase was used in the PCR. A 0.5-kb DNA fragment obtained by digesting the ends on one side of this PCR fragment with *Xho*I was purified, and its 5' end on the blunt end side was phosphorylated with T4 polynucleotide kinase according to an ordinary method. On the other hand, primers represented by SEQ ID NOs: 33 and 34 in the sequence listing were used to perform amplification by PCR with the plasmid pHN159 as a template. The Pfu turbo DNA Polymerase was used in this PCR. A 1.1-kb DNA fragment obtained by digesting the ends on one side of this PCR fragment with *Not*I was purified, and its 5' end on the blunt end side was phosphorylated with T4 polynucleotide kinase according to an ordinary method. These two PCR fragments were simultaneously subcloned into the *Xho*I and *Not*I sites of a plasmid pBluescript II SK(+). Consequently, although the portion of the ligation between blunt ends was the ORF portion of the tetracycline resistance gene, the *Bam*HI recognition site was eliminated without any substitution in encoded amino acids. This plasmid was designated as pHN165.

Next, for eliminating a restriction enzyme recognition site *Sal*I present in the pHN159, the following procedures were performed. At first, primers represented by SEQ ID NOs: 31 and 35 in the sequence listing were used to perform amplification by PCR with the plasmid pHN159 as a template. The Pfu turbo DNA Polymerase was used in this PCR. A 0.8-kb DNA fragment obtained by digesting the ends on one side of this PCR fragment with *Xho*I was purified, and its 5' end on the blunt end side was phosphorylated with T4 polynucleotide kinase according to an ordinary method. On the other hand, primers represented by SEQ ID NOs: 36 and 34 in the sequence listing were used to perform amplification by PCR with the plasmid pHN159 as a template. The Pfu turbo DNA Polymerase was used in this PCR. A 0.8-kb DNA fragment obtained by digesting the ends on one side of this PCR fragment with

*Not*I was purified, and its 5' end on the blunt end side was phosphorylated with T4 polynucleotide kinase according to an ordinary method. These two PCR fragments were simultaneously subcloned into the *Xho*I and *Not*I sites of a plasmid pBluescript II SK(+). Consequently, although the portion of the ligation between blunt ends was the ORF portion of the tetracycline resistance gene, the *Sal*I recognition site was eliminated without any substitution in encoded amino acids. This plasmid was designated as pHN166.

In the last place, the following procedures were performed. A 0.9-kb DNA fragment obtained by doubly digesting the pHN166 with *Sph*I and *Spe*I was subcloned into the *Sph*I and *Spe*I sites of the pHN165. Consequently, a tetracycline resistance gene clone lacking both restriction enzyme recognition sites *Bam*HI and *Sal*I was obtained, and this plasmid was designated as pHN169.

(7) Construction of vector plasmids pHN170 and pHN171

For ligating the genes separated in the above (2) to (6) to construct an expression vector inducible in a bacterium belonging to the genus *Rhodococcus*, the following procedures were performed (Figure 6).

A 1.1-kb DNA fragment obtained by digesting the pHN143 with *Sac*I was subcloned into the *Sac*I site of the pHN136 (subcloned in the orientation of putative *RepB* gene ORF-*tsr* gene ORF-ampicillin resistance gene ORF in the 5' to 3' direction of the DNA). The resulting plasmid was designated as pHN144.

Next, a 1.1-kb DNA fragment obtained by doubly digesting the pHN62 with *Xba*I and *Kpn*I was subcloned into the *Xba*I and *Kpn*I sites of the pHN144. The resulting plasmid was designated as pHN152.

Subsequently, a 1.2-kb DNA fragment obtained by doubly digesting the pHN153 with *Bsr*GI and *Xba*I was subcloned into the *Bsr*GI and *Spe*I sites of the pHN152. The resulting plasmid was designated as pHN154.

Thereafter, a 1.6-kb DNA fragment obtained by doubly digesting the pHN169 with *Xba*I and *Spe*I was subcloned into the *Xba*I site of the pHN154 (subcloned in the orientation of *tsr* gene ORF-tetracycline resistance gene ORF-*ThcA* gene promoter sequence in the 5' to 3'

direction of the DNA). Consequently, a plasmid containing the *PIP* gene placed under the control of the *TipA* gene promoter was constructed and designated as pHN170.

For the high-level expression of a recombinant protein, a ribosome-binding site located downstream of the *TipA* gene promoter was altered into a lambda phage *gene 10*-derived sequence (Gold and Stormo, Methods Enzymol. *185* 89-93 [1990]) allegedly having good translation efficiency (Figure 6). Hereinafter, the procedures will specifically be described.

Primers represented by SEQ ID NOs: 21 and 37 in the sequence listing were used to perform amplification by PCR with the plasmid pHN170 as a template. As a result, a hybrid promoter (hereinafter, indicated by a *TipA-LG10* promoter; indicated by TipA-LG10p in the drawings) consisting of the *TipA* gene promoter and the ribosome-binding site derived from the lambda phage *gene 10* was obtained. This 0.2-kb DNA fragment was doubly digested with restriction enzymes *Bsr*GI and *Nco*I and subcloned into the *Bsr*GI and *Nco*I sites of the pHN170. Consequently, a plasmid containing the *PIP* gene placed under the control of the *TipA-LG10* promoter was constructed and designated as pHN171. Figure 12 shows the *TipA* promoter sequence, and Figure 13 shows the modification of the ribosome-binding site (RBS) sequence for altering the *TipA* promoter into the *TipA-LG10* promoter.

(8) Construction of vector plasmids pTip-NH1, pTip-CH1, pTip-LNH1, and pTip-LCH1

For eliminating the *PIP* gene as a reporter gene from the plasmid described in the above (7) and introducing a multiple-cloning site, the following procedures were performed (Figure 7).

Synthetic oligodeoxyribonucleotides represented by SEQ ID NOs: 38 and 39 in the sequence listing contain a sequence that serves as a multiple-cloning site and have sequences complementary to each other. These two oligodeoxyribonucleotides were mixed in equimolar amounts and treated at 70°C for 10 minutes. The oligodeoxyribonucleotides were cooled to room temperature over 20 minutes and converted into a double strand. As a result, its ends became capable of ligation with a vector doubly digested with *Nco*I and *Spe*I. This synthetic double-stranded DNA (indicated by MCS Linker NNco in the drawings) was subcloned into the *Nco*I and *Spe*I sites of the pHN170. The resulting plasmid was designated as pTip-NH1. Synthetic DNA (indicated by MCS Linker CNco in the drawings) where

synthetic oligodeoxyribonucleotides (containing a sequence that serves as a multiple-cloning site and having sequences complementary to each other) represented by SEQ ID NOs: 40 and 41 in the sequence listing were converted into a double strand in the same way was subcloned into the *Nco*I and *Spe*I sites of the pHN170. The resulting plasmid was designated as pTip-CH1.

The hybrid DNA consisting of the *TipA* gene promoter sequence and the ribosome-binding site derived from the lambda phage *gene 10*, which was described in the above (7), was doubly digested with restriction enzymes *Bsr*GI and *Nco*I and subcloned into the *Bsr*GI and *Nco*I sites of the pTip-NH1 and pTip-CH1, respectively. The resulting plasmids were designated as pTip-LNH1 and pTip-LCH1, respectively.

(9) Construction of vector plasmids pTip-NH2, pTip-CH2, pTip-LNH2, and pTip-LCH2

For altering, into a *Nde*I site, the *Nco*I site, the most upstream site in the multiple-cloning site, in the plasmids pTip-NH1, pTip-CH1, pTip-LNH1, and pTip-LCH1 described in the above (8), the following procedures were performed (Figure 8).

Primers represented by SEQ ID NOs: 21 and 42 in the sequence listing were used to perform amplification by PCR with the plasmid pHN170 as a template. As a result, DNA containing the *TipA* gene promoter was obtained. This 0.2-kb DNA fragment was doubly digested with restriction enzymes *Bsr*GI and *Nde*I and subcloned into the *Bsr*GI and *Nde*I sites of the pHN170. The resulting plasmid was designated as pHN183.

Synthetic oligodeoxyribonucleotides represented by SEQ ID NOs: 43 and 44 in the sequence listing contain a sequence that serves as a multiple-cloning site and have sequences complementary to each other. These two oligodeoxyribonucleotides were mixed in equimolar amounts and treated at 70°C for 10 minutes. The oligodeoxyribonucleotides were cooled to room temperature over 20 minutes and converted into a double strand. As a result, its ends became capable of ligation with a vector doubly digested with *Nde*I and *Spe*I. This synthetic double-stranded DNA (indicated by MCS Linker NNde in the drawings) was subcloned into the *Nde*I and *Spe*I sites of the pHN183. The resulting plasmid was designated as pTip-NH2. Synthetic DNA (indicated by MCS Linker CNde in the drawings) where synthetic oligodeoxyribonucleotides (containing a sequence that serves as a multiple-cloning

site and having sequences complementary to each other) represented by SEQ ID NOs: 45 and 46 in the sequence listing were converted into a double strand in the same way was subcloned in the *Nde*I and *Spe*I sites of the pHN183. The resulting plasmid was designated as pTip-CH2.

Primers represented by SEQ ID NOs: 21 and 47 in the sequence listing were used to perform amplification by PCR with the plasmid pTip-LNH1 as a template. As a result, hybrid DNA consisting of the *TipA* gene promoter and the ribosome-binding site derived from lambda phage *gene 10* was obtained. This 0.2-kb DNA fragment was doubly digested with restriction enzymes *Bsr*GI and *Nde*I and subcloned into the *Bsr*GI and *Nde*I sites of the pTip-NH2 and the pTip-CH2, respectively. The resulting plasmids were designated as pTip-LNH2 and pTip-LCH2, respectively.

Figure 9 collectively shows the maps of the plasmids constructed in the above (8) and (9) and the peripheral sequences of the multiple-cloning sites. In the drawing, a solid line with an arrow head denotes an inverted repeat sequence present in the *TipA* gene promoter; and a dashed line with an arrow head denotes an inverted repeat sequence present in the *ThcA* gene transcription termination sequence. In addition, a -10 region, a -35 region, and RBS generally present in the promoter regions of prokaryotes and important for gene transcription are boxed. The most important SD sequence (Shine and Dalgarno, Eur. J. Biochem. *57* 221-230 [1975]) in RBS is underlined.

(10) Construction of vector plasmids pTip-CH1.1, pTip-CH2.1, pTip-LCH1.1, and pTip-LCH2.1

For bringing a reading frame following the *Xho*I site in the multiple-cloning site in the plasmids pTip-CH1, pTip-CH2, pTip-LCH1, and pTip-LCH2 described in the above (8) and (9) into agreement with the reading frame of a commercially-available pET vector (Novagen), the following procedures were performed (Figure 10).

Primers represented by SEQ ID NOs: 21 and 48 in the sequence listing were used to perform amplification by PCR with the plasmid pTip-CH1 as a template. As a result, DNA containing the *TipA* gene promoter and the multiple-cloning site was obtained. This 0.3-kb DNA fragment was doubly digested with restriction enzymes *Bsr*GI and *Spe*I and subcloned

into the *Bsr*GI and *Spe*I sites of the pTip-CH1. The resulting plasmid was designated as pTip-CH1.1.

Primers represented by SEQ ID NOs: 21 and 48 in the sequence listing were used to perform amplification by PCR with the plasmid pTip-CH2 as a template. As a result, DNA containing the *TipA* gene promoter and the multiple-cloning site was obtained. This 0.3-kb DNA fragment was doubly digested with restriction enzymes *Bsr*GI and *Spe*I and subcloned into the *Bsr*GI and *Spe*I sites of the pTip-CH1. The resulting plasmid was designated as pTip-CH2.1.

Primers represented by SEQ ID NOs: 21 and 48 in the sequence listing were used to perform amplification by PCR with the plasmid pTip-LCH1 as a template. As a result, DNA containing the *TipA-LG10* gene promoter and the multiple-cloning site was obtained. This 0.3-kb DNA fragment was doubly digested with restriction enzymes *Bsr*GI and *Spe*I and subcloned into the *Bsr*GI and *Spe*I sites of the pTip-CH1. The resulting plasmid was designated as pTip-LCH1.1.

Primers represented by SEQ ID NOs: 21 and 48 in the sequence listing were used to perform amplification by PCR with the plasmid pTip-LCH2 as a template. As a result, DNA containing the *TipA-LG10* gene promoter and the multiple-cloning site was obtained. This 0.3-kb DNA fragment was doubly digested with restriction enzymes *Bsr*GI and *Spe*I and subcloned into the *Bsr*GI and *Spe*I sites of the pTip-CH1. The resulting plasmid was designated as pTip-LCH2.1.

(11) Construction of vector plasmids pHN172 and pHN173

For investigating whether the induction of expression was strictly regulated, a plasmid for a control experiment as described below was constructed (Figure 11).

A 1.6-kb DNA fragment obtained by doubly digesting the pHN169 with *Xba*I and *Spe*I was subcloned into the *Xba*I site of the pHN144 (subcloned in the orientation of *tsr* gene ORF-tetracycline resistance gene ORF-ampicillin resistance gene ORF in the 5' to 3' direction of the DNA). The resulting plasmid was designated as pHN172.

Next, a 1.2-kb DNA fragment obtained by doubly digesting the pHN153 with *Bsr*GI and *Xba*I was subcloned into the *Bsr*GI and *Spe*I sites of the pHN144. The resulting plasmid

was designated as pHN164. Subsequently, a 1.6-kb DNA fragment obtained by doubly digesting the pHN169 with *Xba*I and *Spe*I was subcloned into the *Xba*I site of the pHN164 (subcloned in the orientation of *tsr* gene ORF-tetracycline resistance gene ORF-ampicillin resistance gene ORF in the 5' to 3' direction of the DNA). The resulting plasmid was designated as pHN173.

The pHN170 carries a gene cassette where three elements, the *TipA* gene promoter, the *PIP* ORF located downstream of the promoter, and the *ThcA* gene transcription termination sequence located downstream of the *PIP* ORF, are connected (hereinafter, indicated by an expression cassette) as well as a gene cassette where two elements, the *ThcA* gene promoter and the *TipA* gene located downstream of the promoter, are connected (hereinafter, indicated by an inducer cassette). The pHN173 has only the expression cassette, and the pHN 172 lacks both cassettes.

(12) Transformation of bacterium belonging to genus *Rhodococcus*

A *Rhodococcus erythropolis* strain JCM3201 was subjected to shaking culture at 30°C in 100 ml of a LB medium until reaching the logarithmic growth phase. The resulting culture solution was cooled on ice for 30 minutes and centrifuged to recover the bacterial cells. These bacterial cells were supplemented with 100 ml of ice-cold sterile water, then sufficiently stirred, and centrifuged again to recover the bacterial cells. These bacterial cells were supplemented with 100 ml of ice-cold 10% glycerol solution, then sufficiently stirred, and centrifuged to recover the bacterial cells. After this washing with ice-cold 10% glycerol solution was repeated again, the resulting bacterial cells were suspended in 5 ml of ice-cold 10% glycerol solution. A 400-μl aliquot thereof was dispensed and momentarily frozen with liquid nitrogen. The bacterial cells were stored at -80°C until use. The bacterial cells stored at -80°C were thawed on ice and supplemented with 3 μl of the plasmid pHN170 or pHN172 or pHN173 (each approximately 300 ng). This mixture solutions of the bacterial cell and the DNA were transferred to electroporation cuvettes (manufactured by Bio-Rad; 0.2-cm-gap cuvettes), to which electric pulses were respectively applied with a gene transfer apparatus Gene Pulser II (also manufactured by Bio-Rad) at electric field strength of 12.5 kV/cm, with the pulse controller set to capacitance of 25 μF and external resistance of 400 Ω. The

mixture solutions of the bacterial cell and the DNA treated with electric pulses were mixed with 1 ml of a LB medium and cultured at 30°C for 4 hours to recover the bacterial cells. The bacterial cells were spread onto LB agar media (agar concentration: 1.8%) containing 20 μg/ml tetracycline and cultured at 30°C for 3 days to yield the respective transformants.

[Example 1]

Experimental Method

At first, approaches used in experiments described in Examples 2 to 12 below are enumerated.

All plasmids were constructed according to an ordinary method (Sambrook et al., Molecular Cloning: a laboratory manual, 2nd edition [1989], Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.). All polymerase chain reaction methods (hereinafter, abbreviated to PCR; Saiki et al., Science *239* 487-491 [1988]) employed the Pfu turbo (manufactured by STRATAGENE). DNA fragments excised from the plasmids were subjected to 1.0% agarose gel electrophoresis, and DNA fragments of interest were excised from the gels and purified using the QIA quick Gel Extraction Kit (manufactured by QIAGEN) according to the instruction. Procedures for separating the genomic DNAs of a *Streptomyces coelicolor* strain A3(2) and a *R. erythropolis* strain DSM313 and for purifying plasmid DNA from a bacterium belonging to the genus *Rhodococcus* were performed in the same way as described in Reference Example 1. The genomic DNA of an *Escherichia coli* strain ER2508 (manufactured by New England Biolabs) was purified using the QIAGEN RNA/DNA Mini Kit (manufactured by QIAGEN) according to the instruction. When the 5' ends of the DNA fragments need to be phosphorylated, T4 polynucleotide kinase (manufactured by Toyobo) was used. A DNA sequencer ABI PRISM(R) 3100 Genetic Analyzer (manufactured by ABI) was used in the determination of nucleotide sequences. T4 DNA ligase (manufactured by New England Biolabs) was employed in ligase reaction.

Main plasmids and strains used are shown in Tables 1 and 2. A bacterium belonging to the genus *Rhodococcus*, a *Streptomyces coelicolor* strain A3(2), and *Escherichia coli* were cultured in Luria Broth (LB; 1% Bacto trypton, 0.5% Bacto yeast extract, and 1% sodium chloride). Although procedures for constructing the competent cell of a bacterium belonging

to the genus *Rhodococcus* and for its transformation were described in Reference Example 1, the construction of the competent cell of a bacterium belonging to the genus *Rhodococcus* that was transformed with a plasmid in advance was performed from bacterial cells cultured in a LB medium containing an appropriate antibiotic. For selecting a transformant, tetracycline (at concentrations of 8 μg/ml in a liquid medium and 20 μg/ml in a solid medium), chloramphenicol (34 μg/ml), and ampicillin (50 μg/ml) were used.

When proline iminopeptidase (hereinafter, PIP) or green fluorescent protein (hereinafter, GFP) was expressed in a bacterium belonging to the genus *Rhodococcus* with an inducible vector, the transformant of the bacterium belonging to the genus *Rhodococcus* was cultured at 30°C in a LB medium containing an appropriate antibiotic, and supplemented with thiostrepton (solvent: dimethylsulfoxide) at the final concentration of 1 μg/ml at the point in time when an optical density measured at a wavelength of 600 nm (O.D. 600) reached 0.6, followed by additional 16-hour culture. When PIP or GFP was expressed with a constitutive vector, the transformant of the bacterium belonging to the genus *Rhodococcus* was cultured at 30°C in a LB medium containing an appropriate antibiotic to an O.D. 600 of 2.0.

Hereinafter, procedures for measuring peptidase activity of PIP will be described in detail. The culture solution of the bacterium belonging to the genus *Rhodococcus* where the PIP was expressed as described above was brought up to 200 μl with a LB medium containing an appropriate antibiotic (8 μg/ml) and heated at 60°C for 1 minute. To the solution, 2 μl of H-Pro-βNA (100 mM; solvent: dimethylsulfoxide) was added as a substrate for the PIP and incubated at 60°C for 20 minutes (optimum temperature for PIP: 60°C). For monitoring βNA liberated from the H-Pro-βNA by PIP hydrolysis, 134 μl of a Fast Garnet GBC Salt solution (manufactured by Wako Pure Chemical Industries; concentration: 0.5 mg/ml; solvent: 1M sodium acetate buffer (pH 4.2) and 10% Triton X-100) was added as a coloring agent. The above-described mixture solution turns yellow without the expression of PIP or turns red with the expression of PIP. The absorbance at 550 nm (A550) of the developed red color was measured with an absorption spectrophotometer to quantify PIP activity. The measurement was performed after the mixture solution supplemented with Fast Garnet GBC Salt was diluted with 666 μl of sterile water.

In the measurement at 550 nm, the optical density of the cell was also measured. Accordingly, the optical density of the cell at 550 nm (O.D. 550) was separately measured. A value corrected by subtracting a value corresponding to O.D. 550 used at the time of measurement from a value of A550 is used as Ac550, that is, calculated by Ac550=A550-O.D. 550xAmount (ml) of culture solution used in activity measurement of PIP. A unit value is meant to a "value of Ac550 per ml of the culture solution for O.D. 600=1 obtained in 20-minute measurement" and calculated by "AC550÷Amount (ml) of culture solution used in activity measurement of PIP÷O.D. 600."

[Example 2]

Separation of novel endogenous plasmid pRE8424 present in *R. erythropolis*

The present inventors searched for a novel endogenous plasmid present in *R. erythropolis* and found small circular plasmids from four strains, *R. erythropolis* JCM2893, *R. erythropolis* JCM2894, *R. erythropolis* DSM43200, and *R. erythropolis* DSM8424. These plasmids were designated as pRE2893, pRE2894, pRE43200, and pRE8424, respectively.

Of these plasmids, the DNA sequences of the plasmids pRE2893, pRE2894, and pRE43200 were partially determined. As a result, they had a sequence nearly identical to that of the pRE2895 (see Reference Example 1), which had been separated previously by the present inventors from an *R. erythropolis* strain JCM2895. The pRE2895 has, as *RepAB* operon, genes encoding RepA and RepB proteins, which are involved in the replication of plasmids. These proteins are highly similar to RepA and RepB proteins encoded by a plasmid pAL5000 separated from *Mycobacterium fortuitum*, and it has been suggested that the pRE2895 and the pAL5000 autonomously replicate in a similar mode (Stolt and Stoker, Microbiology *142* 2795-2802 [1996]; Reference Example 1). Although the modes of replication of the pRE2895 and the pAL5000 have not been elucidated, it has been considered that the RepA proteins of both plasmids have homology to the Rep protein of a plasmid ColE2, so that they have the "θ type" mode of autonomous replication as with the plasmid ColE2 (Hiraga et al., J. Bacteriol. *176* 7233-7243 [1994]).

On the other hand, the pRE8424 had a DNA sequence completely different from that of the pRE2895 (SEQ ID NO: 90 in the sequence listing; Figure 1). This plasmid carries six

open reading frames (ORFs; ORF1 to ORF6). Of these ORFs, the ORF6 encoded a protein (Figure 14) with high homology to proteins encoded by the *Rep* genes of a group of plasmids that autonomously replicate in the rolling circle mode (Khan, Microiol. Mol. Biol. Rev. *61* 442-455 [1997]). The pRE8424 had especially high homology to pAP1 derived from *Arcanobacterium pyrogenes* (Billington et al., J. Bacteriol. *180* 3233-3236 [1998]), pIJ101 derived from *Streptomyces lividans* (Kendall et al., J. Bacteriol. *170* 4634-4651 [1988]), pJV1 derived from *Streptomyces phaeochromogenes* (Servin-Gonzalez et al., Microbiology *141* 2499-2510 [1995]), pBL1 derived from *Brevibacterium lactofermentum* (Fernandez-Gonzalez et al., J. Bacteriol. *176* 3154-3161 [1994]), and pSN22 derived from *Streptomyces nigrifaciens* (Kataoka et al., Plasmid *32* 55-69 [1994]) (Figure 15). All of these plasmids belong to the pIJ101/pJV1 family among rolling-circle-replication plasmids (Khan, Microiol. Moi. Biol. Rev. *61* 442-455 [1997]). This has suggested the possibility that the pRE8424 is also a rolling-circle-replication plasmid belonging to this family. Hereinafter, *ORF6* is referred to as *Rep*.

In general, the autonomous replication of the rolling-circle-replication plasmid in a host cell requires DNA sequences that serve as a double-stranded origin (hereinafter, DSO) and a single-stranded origin (hereinafter, SSO), in addition to the foregoing *Rep*. The present inventors have constructed a variety of pRE8424 mutants with which *R. erythropolis* was then transformed, and have conducted various analyses to identify the localization of DSO and SSO sequences (Figure 14). Although the DSO has been considered to be located within nucleotide Nos. 5514 to 5970 in SEQ ID NO: 90 in the sequence listing, comparison with the DSO sequences of other rolling-circle-replication plasmids has indicated that a sequence of nucleotide Nos. 5705 to 5734 in SEQ ID NO: 90 in the sequence listing is most important for the function of the DSO (Figure 16). The identified SSO sequence is shown in Figure 17. SSO sequences generally have a high secondary structure such as the stem-loop structure. Plasmids in the pIJ101/pJV1 family often have a consensus sequence consisting of, for example, TAGCGT, in the loop region of the stem-loop structure. The SSO of the pRE8424 also has a high secondary structure and has a TAGCGG sequence in the loop region (Figure 17).

The present inventors have found that the derived plasmids of pRE8424 having a mutation in the above-described TAGCGG sequence are accumulated as single-stranded DNAs in large amounts in the cells of *R. erythropolis*. The accumulation of single-stranded DNA is a hallmark of rolling-circle-replication plasmids (Khan, Microiol. Moi. Biol. Rev. *61* 442-455 [1997]) and as such, the pRE8424 has been shown to autonomously replicate in the rolling circle mode.

A 2.0-kb region containing *Rep*, DSO, and SSO, that is, a region of nucleotide Nos. 3845 to 5849 in SEQ ID NO: 90 in the sequence listing, was sufficient for the autonomous replication of the derived plasmids of pRE8424 in *R. erythropolis* used as a host cell (see Example 3 below).

Figure 14 shows the map of the pRE8424. In Figure 14, main restriction enzyme recognition sites are shown, and six ORFs are indicated by arrows. The locations of DSO and SSO are indicated by boxes.

Figure 15 shows the amino acid sequences of five motifs (Motif IV, Motif I, Motif II, Motif III, and C-terminal motif; see Billington et al., J. Bacteriol. *180* 3233-3236 [1998]) that are conserved in Rep proteins from the pRE8424, the pAP1, the pBL1, the pJV1, the PIJ101, and the pSN22. A numeric denotes the number of an amino acid residue located between the motifs, that is, the number of an amino acid residue in a gap. A perfectly conserved amino acid residue, a highly conserved region, and a relatively conserved region are indicated by an asterisk (*), two dots (:), and one dot (.), respectively. A tyrosine residue allegedly important for the function of the Rep protein is boxed.

Figure 16 shows an especially conserved DNA region in sequences likely to be the DSOs of the pRE8424, the pAP1, the pBL1, the pJV1, the pIJ101, and the pSN22. In addition, a GG dinucleotide particularly important for the function of the DSO is underlined (see Billington et al., J. Bacteriol. *180* 3233-3236 [1998]).

Figure 17 shows the SSO of the pRE8424, that is, a sequence of nucleotide Nos. 5268 to 5538 in SEQ ID NO: 90 in the sequence listing, and a possible secondary structure. The prediction of the secondary structure was performed by the mfold program, version 3.0 (Michael Zuker, Washington University, St. Louis, Mo.;

http://www.bioinfo.rpi.edu/applications/mfold/old/dna/form1.cgi). The above-described TAGCGG sequence is indicated by a filled-in circle.

[Example 3]

Construction of pHN372

For eliminating an unnecessary restriction enzyme recognition site *Bam*HI present in the 2.0-kb region essential for the autonomous replication of the pRE8424, the following procedures were performed.

Synthetic oligodeoxyribonucleotide primers (hereinafter, abbreviated to primers) represented by SEQ ID NOs: 57 (sHN389) and 58 (sHN390) in the sequence listing were used to perform DNA amplification by PCR with the pRE8424 as a template. The obtained 1.0-kb fragment contains the 5' end portion of the *Rep*. The 5' ends of this fragment were phosphorylated, and the fragment was introduced into the *Hinc*II site of pBluescript II SK(+) (manufactured by STRATAGENE) to yield a plasmid, which was designated as pHN371. Primers represented by SEQ ID NOs: 59 (sHN391) and 60 (sHN321) in the sequence listing were used to perform DNA amplification by PCR with the pRE8424 as a template. The obtained 1.0-kb fragment contains the 3' end portion of the *Rep*. This fragment was digested with *Bam*HI, and its 5' ends were phosphorylated. The fragment was introduced into the *Eco*RV/*Bgl*II sites of the pHN371. The resulting plasmid was designated as pHN372. The pHN372 has the 2.0-kb region essential for the autonomous replication of the pRE8424 and lacks the *Bam*HI site present in the pRE8424. The elimination of the *Bam*HI site did not affect the function of the autonomous replication of the pRE8424.

[Example 4]

Construction of pHN346

In the construction of the vectors shown in Reference Example, only a tetracycline resistance gene was developed as a selection marker for the transformant of a bacterium belonging to the genus *Rhodococcus*. However, the transformation of the bacterium belonging to the genus *Rhodococcus* with several plasmids requires newly developing resistance genes to other antibiotics. The present inventors have found that a *R. erythropolis* strain DSM313 is resistant to chloramphenicol and have decided to separate a gene that

imparts resistance. Two chloramphenicol resistance genes have already been separated from bacteria belonging to the genus *Rhodococcus* (*cmrA* gene and *cmr* gene), and these genes have high homology to each other (De Mot et al., Microbiology *143* 3137-3147 [1997]; and Desomer et al., Mol. Microbiol. *6* 2377-2385 [1992]).

Because the chloramphenicol resistance gene of the *R. erythropolis* strain DSM313 was expected to be homologous to these genes, primers represented by SEQ ID NOs: 61 (sHN335) and 62 (sHN336) in the sequence listing were used to perform DNA amplification by PCR with the genomic DNA of the *R. erythropolis* strain DSM313 as a template. The primers were designed on the basis of sequences having the highest homology in the *cmrA* gene and the *cmr* gene. As a result, a 0.7-kb amplified band was confirmed. When the DNA sequence of this PCR product was determined, the sequence had considerably high homology to that of the *cmrA* gene. Primers represented by SEQ ID NOs: 63 (sHN349) and 64 (sHN351) in the sequence listing were designed based on the determined sequence, and the full-length chloramphenicol resistance gene of the *R. erythropolis* strain DSM313 was separated by inverse PCR (Ochman et al., Genetics *120* 621-623[1988]). DNA used as a template was obtained by cleaving 0.1 μg of the genomic DNA of the *R. erythropolis* strain DSM313 with *Sal*I and circularizing the cleaved genomic DNA by self-ligation with ligase. The obtained PCR product was 2.3 kb, and the full DNA sequence of this fragment was determined. One ORF was present in this fragment, and this gene was designated as *ChlA* (indicated by Chl^r in the drawings).

Primers represented by SEQ ID NOs: 65 (sHN361) and 66 (sHN362) in the sequence listing were used to perform DNA amplification by PCR with the genomic DNA of the *R. erythropolis* strain DSM313 as a template. The obtained 0.5-kb fragment contains the 5' end portion of the chloramphenicol resistance gene. This fragment was digested with *Sac*I, and its 5' ends were phosphorylated. On the other hand, primers represented by SEQ ID NOs: 67 (sHN363) and 68 (sHN364) in the sequence listing were used to perform DNA amplification by PCR with the genomic DNA of the *R. erythropolis* strain DSM313 as a template. The obtained 1.3-kb fragment contains the 3' end portion of the chloramphenicol resistance gene. This fragment was digested with *Spe*I, and its 5' ends were phosphorylated. These two DNA

fragments were simultaneously introduced into the *SacI/Spe*I sites of pBluescript II SK(+) to yield a plasmid, which was designated as pHN346. The pHN346 has the full-length chloramphenicol resistance gene but lacks the *Eco*RI site originally present in the ORF (without any change in the amino acid sequences of encoded proteins).

[Example 5]

Construction of inducible expression vectors having proline iminopeptidase (PIP) gene as a reporter gene; Construction of pHN171, pHN379, pHN348, and pHN380

A 1.8-kb fragment containing the chloramphenicol resistance gene was excised from the pHN346 (Example 4) with *Xba*I and *Spe*I and introduced into the *Xba*I site of pHN154 (Japanese Patent Application No. 2002-235008). The resulting plasmid was designated as pHN347. A 1.1-kb fragment was excised from the pHN171 (see Reference Example) with *Bsr*GI and *Spe*I and introduced into the *BsrGI/Spe*I sites of the pHN347. The resulting plasmid was designated as pHN348.

Although both of the pHN171 and the pHN348 were expression vectors where a *PIP* gene, a reporter gene, was introduced into the MCS of the pTip vector (see Reference Example), the difference between them is only in a transformation marker: a tetracycline resistance gene for the pHN171 and a chloramphenicol resistance gene for the pHN348. In any of the plasmids, a ribosome-binding site sequence originally located downstream of the *TipA* gene promoter (TipA-RBS) is altered into a bacteriophage *gene 10*-derived ribosome-binding site sequence having good translation efficiency (*TipA-LG10* promoter; see Reference Example). A 6xHis tag is adapted to be attached to the C terminus of PIP in order to facilitate protein purification. The 6xHis tag is a consecutive sequence consisting of six consecutive histidine residues, and a protein fused with this tag exhibits high affinity for a nickel ion or the like. Thus, the protein is readily purified by metal chelate chromatography that employs the nickel ion or the like (Crowe et al., Methods Mol. Biol. *31* 371-387 [1994]).

For altering a 1.9-kb region essential for the autonomous replication of plasmids derived from pRE2895 in the DNA sequences of the above-described pHN171 and pHN348 into a 2.0-kb region essential for the autonomous replication of plasmids derived from pRE8424, the following procedures were performed.

Primers represented by SEQ ID NOs: 69 (sHN368) and 70 (sHN373) in the sequence listing were used to perform DNA amplification by PCR with the pHN171 as a template. The obtained 0.2-kb fragment contains the 5' end portion of the thiostrepton resistance gene (*tsr* gene; indicated by Thio^r in the drawings) (Bibb et al., Mol.Gen.Genet. *199* 26-36 [1985]). This fragment was digested with *Bsr*GI and *Cla*I and introduced into the *Bsr*GI/*Cla*I sites of the pHN171 and the pHN348, respectively. The resulting plasmids were designated as pHN357 and pHN358, respectively. A 2.0-kb fragment containing a region essential for the autonomous replication of plasmids derived from pRE8424 was excised from the pHN372 (Example 3) with *Bsr*GI and *Hpa*I and introduced into the *Bsr*GI/*Hpa*I sites of the pHN357 and the pHN358, respectively. The resulting plasmids were designated as pHN379 and pHN380, respectively.

[Example 6]

Construction of pTip vectors

The process of constructing eight pTip vectors by introducing MCS instead of the *PIP* gene of the plasmids pHN171, pHN348, pHN379, and pHN380 (Example 5) will be illustrated. Four (pTip-RT1, pTip-RT2, pTip-RC1, and pTip-RC2, which will be described below) of the pTip vectors constructed this time are different in a DNA region necessary for the autonomous replication of a plasmid in a bacterium belonging to the genus *Rhodococcus* from the pTip vectors described in Reference Example 1, and do not cause incompatibility with all of the pTip vectors described in Reference Example 1 in a bacterium belonging to the genus *Rhodococcus* (described below). Alternatively, the remaining four (pTip-QT1, pTip-QT2, pTip-QC1, and pTip-QC2, which will be described below) are different in a part of a MCS sequence from the pTip vectors described in Reference Example 1.

Synthetic oligodeoxyribonucleotides represented by SEQ ID NOs: 71 and 72 in the sequence listing contain a sequence that serves as a MCS site and have sequences complementary to each other. These two oligodeoxyribonucleotides were mixed in equimolar amounts and treated at 70°C for 10 minutes. The oligodeoxyribonucleotides were cooled to room temperature over 20 minutes and converted into a double strand (MCS type 1). As a result, its ends became capable of ligation with a vector doubly digested with *Nco*I and

*Spe*I. This synthetic double-stranded DNA was subcloned into the *Nco*I/*Spe*I sites of the pHN379 and the pHN380, respectively. The resulting plasmids were designated as pTip-RT1 and pTip-RC1, respectively. Synthetic oligodeoxyribonucleotides represented by SEQ ID NOs: 73 and 74 in the sequence listing were converted into a double strand (MCS type 2) in the same way. On the other hand, a 0.2-kb fragment containing the *TipA* gene promoter and LG10-RBS was excised from the pTip-LNH2 (see Reference Example 1) with *Bsr*GI and *Nde*I. These two DNA fragments were simultaneously introduced into the *Bsr*GI/*Spe*I sites of the pHN379 and the pHN380, respectively. The resulting plasmids were designated as pTip-RT2 and pTip-RC2, respectively. A 0.3-kb fragment containing the *TipA* gene promoter, the LG10-RBS, and the MCS type 1 was excised from the pTip-RT1 with *Bsr*GI and *Spe*I and introduced into the *Bsr*GI/*Spe*I sites of the pHN171 and the pHN348, respectively. The resulting plasmids were designated as pTip-QT1 and pTip-QC1, respectively. A 0.3-kb fragment containing the *TipA* gene promoter, the LG10-RBS, and the MCS type 2 was excised from the pTip-RT2 with *Bsr*GI and *Spe*I and introduced into the *Bsr*GI/*Spe*I sites of the pHN171 and the pHN348, respectively. The resulting plasmids were designated as pTip-QT2 and pTip-QC2, respectively.

Figure 18-1 shows the maps of the pTip vectors (pTip-QT1, pTip-QT2, pTip-RT1, pTip-RT2, pTip-QC1, pTip-QC2, pTip-RC1, and pTip-RC2). In the drawing, Thio^r denotes a thiostrepton resistance gene; Tuf1p denotes a *Tuf1* gene promoter; Tet^r denotes a tetracycline resistance gene; Chl^r denotes a chloramphenicol resistance gene (each pTip vector has any one of Tuf1p-Tet^r or Chl^r); ALDHp denotes a *ThcA* promoter that allows the transcription of the *TipA* gene (*TipA*); Amp^r denotes an ampicillin resistance gene; ColE1 denotes the replication origin of *Escherichia coli*; ALDHt denotes a *ThcA* gene transcription termination sequence; MCS denotes a multiple-cloning site (each pTip vector has any one of MCS type 1 or MCS type 2); TipAp denotes a *TipA* gene promoter; TipA-LG10p denotes a *TipA-LG10* promoter; *RepA&B* denotes a region essential for the autonomous replication of plasmids derived from pRE2895 in *R. erythropolis*; and *Rep* denotes a region essential for the autonomous replication of plasmids derived from pRE8424 in *R. erythropolis* (each pTip vector has any one of *RepA&B* or *Rep*). Incidentally, a diagram for pNit vectors (described below) described in

Example 9 is indicated in a right half in the drawing, and the same symbols are used to designate the same components.

Figure 20 shows the DNA sequence of *TipA-LG10* promoter-MCS-*ThcA* gene terminator. In the drawing, a solid line with an arrow head denotes an inverted repeat sequence present in the *TipA* gene promoter; and a dashed line with an arrow head denotes an inverted repeat sequence present in the *ThcA* gene transcription termination sequence. In addition, a -10 region and a -35 region generally present in the promoter regions of prokaryotes and important for gene transcription are boxed. A TATAAT sequence that is boxed shows an introduced mutation in the construction of a *Nit* promoter from the *TipA* gene promoter (described in detail in Example 7).

[Example 7]

Construction of pHN231

At first, the present inventors have decided that a mutation is introduced into the *TipA* gene promoter to alter the inducible promoter to a constitutive promoter. It has long been known that a thiostrepton-TipA protein complex binds to the "inverted repeat" region in the *TipA* gene promoter sequence to promote the transcription of its own gene (Holmes et al., EMBO J. *12* 3183-3191 [1993]). Thus, the present inventors have speculated that the mutations in the inverted repeat sequence would bring about some change in the transcription activity of the *TipA* gene promoter, and have therefore constructed a variety of *TipA* gene promoter mutants. Of these mutants, in a mutant where a mutation was introduced into the so-called -10 region of the *TipA* gene promoter (Fenton and Gralla. Proc. Natl. Acad. Sci. USA *98* 9020-9025 [2001]) (Figure 19; mutation from CAGCGT to TATAAT), the expression of the reporter gene was observed even in the absence of thiostrepton (Figure 20; described in detail in Example 10). This DNA sequence consisting of TATAAT is a sequence frequently found in a -10 region in a DNA sequence that functions as a considerably strong promoter in *Escherichia coli*. The conclusion drawn from the above view is that this mutated *TipA* gene promoter is a constitutive promoter. This constitutive promoter was designated as a *Nit* (non-inducible TipA; indicated by *Nitp* in the drawings) promoter.

The process of constructing the *Nit* promoter will be illustrated below. Primers represented by SEQ ID NOs: 75 (sHN217) and 76 (sHN218) in the sequence listing were used to perform DNA amplification by inverse PCR with the pHN150u (see Reference Example 1) as a template. The pHN150u is a plasmid obtained by cloning a wild-type *TipA* gene promoter into the MCS of pBluescript II SK(+). The 5' ends of the above-described two primers were respectively phosphorylated. This inverse PCR fragment was circularized by self-ligation through ligase reaction. The resulting plasmid was designated as pHN231. The pHN231 assumes the form where the *Nit* promoter was cloned into the MCS of the pBluescript II SK(+).

[Example 8]

Construction of inducible expression vectors having proline iminopeptidase (PIP) gene as a reporter gene; Construction of pHN407, pHN385, pHN409, and pHN389

Primers represented by SEQ ID NOs: 77 (sHN395) and 78 (sHN396) in the sequence listing were used to perform DNA amplification by PCR with the pTip-NH1 (see Reference Example 1) as a template. The obtained 1.6-kb fragment contains the tetracycline resistance gene. This fragment was digested with *Hpa*I and *Kpn*I and introduced into the *Hpa*I/*Kpn*I sites of the pHN379 (Example 5). The resulting plasmid was designated as pHN381. Primers represented by SEQ ID NOs: 79 (sHN397) and 80 (sHN398) in the sequence listing were used to perform DNA amplification by PCR with the pHN346 (Example 4) as a template. The obtained 1.8-kb fragment contains the chloramphenicol resistance gene. This fragment was digested with *Hpa*I and *Kpn*I and introduced into the *Hpa*I/*Kpn*I sites of the pHN380 (Example 5). The resulting plasmid was designated as pHN382. A 0.2-kb fragment containing the *Nit* promoter was excised from the pHN231 (Example 7) with *Bsr*GI and *Nco*I and introduced into the *Bsr*GI/*Nco*I sites of the pHN381 and the pHN382, respectively. The resulting plasmids were designated as pHN383 and pHN387, respectively. Primers represented by SEQ ID NOs: 81 (sHN147) and 82 (sHN376) in the sequence listing were used to perform DNA amplification by PCR with the pHN231 (Example 7) as a template. The obtained 0.2-kb fragment lacks the RBS portion of the *Nit* promoter. This fragment was digested with *Bsr*GI and *Xba*I and introduced into the *Bsr*GI/*Xba*I sites of the pHN381 and the

pHN382, respectively. The resulting plasmids were designated as pHN385 and pHN389, respectively. The hybrid DNA between this *Nit* promoter (except for the RBS portion) and LG10RBS was designated as a *Nit-LG10* promoter. Primers represented by SEQ ID NOs: 83 (sHN388) and 84 (sHN120) in the sequence listing were used to perform DNA amplification by PCR with the pHN171 as a template. The obtained 1.9-kb fragment contains the *RepAB* operon derived from the pRE2895. This fragment was digested with *Bsr*GI and *Hpa*I and introduced into the *Bsr*GI/*Hpa*I sites of the pHN387 and the pHN389, respectively. The resulting plasmids were designated as pHN407 and pHN409, respectively.

In addition, for constructing a plasmid for a control experiment, a 0.2-kb *Nit* promoter was excised from the pHN387 with *Bsr*GI and *Nco*I. This DNA fragment was introduced into the *Bsr*GI/*Nco*I sites of the pHN380 (Example 5). The resulting plasmid was designated as pHN410.

[Example 9]

Construction of pNit vectors

The process of constructing eight pNit vectors by introducing MCS instead of the *PIP* gene of the plasmids pHN407, pHN385, pHN409, and pHN389 (Example 8) will be illustrated.

A 2.2-kb fragment was excised from the pTip-RT1 (Example 6) with *Nco*I and *Kpn*I and introduced into the *Nco*I/*Kpn*I sites of the pHN407, the pHN385, the pHN409, and the pHN389, respectively. The resulting plasmids were designated as pNit-QT1, pNit-RT1, pNit-QC1, and pNit-RC1, respectively. Primers represented by SEQ ID NOs: 81 (sHN147) and 85 (sHN160) in the sequence listing were used to perform DNA amplification by PCR with the pHN385 (Example 8) as a template. The obtained 0.2-kb fragment contains the *Nit-LG10* promoter. This fragment was digested with *Bsr*GI and *Nde*I. On the other hand, a 2.0-kb fragment containing the MCS type 2, the ampicillin resistance gene, and the *Col*E1 was excised from the pTip-RT2 (Example 6) with *Nde*I and *Kpn*I. These two DNA fragments were simultaneously introduced into the *Bsr*GI/*Kpn*I sites of the pHN407, the pHN385, the pHN409, and the pHN389 (Example 8), respectively. The resulting plasmids were designated as pNit-QT2, pNit-RT2, pNit-QC2, and pNit-RC2, respectively.

Figure 18-2 shows the maps of the pNit vectors (pNit-QT1, pNit-QT2, pNit-RT1, pNit-RT2, pNit-QC1, pNit-QC2, pNit-RC1, and pNit-RC2). Symbols and so on are as described in Example 6.

[Example 10]

Expression of *PIP* gene from *TipA* gene promoter and *Nit* promoter

Using the plasmids pHN380, pHN410, pHN381, pHN387, and pHN389, the mode of gene expression from the pTip and pNit vectors was observed. Hereinafter, its process and result will be illustrated.

At first, a *R. erythropolis* strain JCM3201 was transformed with the pHN380, the pHN410, the pHN381, the pHN387, and the pHN389. These transformants were used to measure the peptidase activity of PIP. The result is shown in Figure 20.

In Figure 20, the designations and simple features of the plasmids used in transformation were shown. A filled-in bar and a shaded bar denote the peptidase activity of PIP with the treatment of the transformant with thiostrepton and without the treatment of the transformant with thiostrepton, respectively. Thiostrepton-controlled gene expression works in the transformant obtained with the pHN380 (having, in the pTip vector skeleton, the gene cassette consisting of *TipA-LG10* promoter-*PIP*), whereas thiostrepton-controlled gene expression does not work in the transformant obtained with the pHN410 (having, in the pTip vector skeleton, the gene cassette consisting of *Nit* promoter-*PIP*). Alternatively, the pHN387 is a plasmid assuming the form where the thiostrepton resistance gene and the gene cassette consisting of *ThcA* gene promoter-*TipA* gene have been removed from the pHN410. The *PIP* gene was also expressed in the transformant obtained with this plasmid even in the absence of thiostrepton. This means that gene expression from the *Nit* promoter occurs even in the absence of the TipA protein. Results of using the transformants obtained with the pHN387 and the pHN389 have suggested that a RBS sequence is not involved in thiostrepton-controlled gene expression. The pHN381 was a plasmid obtained by substituting a *TipA-LG10* promoter for the *Nit-LG10* promoter of the pHN389. The expression of the *PIP* gene is not constitutive in the transformant obtained with the pHN381.

The above view has revealed that the *Nit* promoter and the *Nit-LG10* promoter are constitutive promoters under which the expression of a *PIP* gene does not require a TipA protein.

It was confirmed that the *PIP* can also be expressed from the pTip and pNit vectors at 4°C in addition to at 30°C.

[Example 11]

Comparison of regions essential for autonomous replication of plasmids derived from pRE2895 and pRE8424

Using the pTip and pNit vectors, the features of regions essential for the autonomous replication of plasmids derived from pRE2895 and pRE8424 was investigated.

At first, the transformation efficiency of the pNit-QC2 and the pNit-RC2 for *R. erythropolis* JCM 3201, *R. fascians* JCM10002, *R. opacus* DSM44193, *R. ruber* JCM3205, and *R. rhodochrous* JCM3202 was investigated. The result is shown in Table 3. Table 3 shows the number of colonies appearing on a solid medium containing chloramphenicol in the case that 1 μg each of the plasmid DNA was used in transformation. This result has demonstrated that *R. erythropolis* JCM 3201, *R. fascians* JCM10002, and *R. opacus* DSM44193 can be transformed with both of the pNit-QC2 and the pNit-RC2, though with a difference in efficiency. Neither *R. ruber* JCM3205 nor *R. rhodochrous* JCM3202 produced a transformant.

Next, *R. erythropolis* JCM3201, *R. fascians* JCM10002, and *R. opacus* DSM44193 were transformed with the pHN409 and the pHN389 (Example 9). The difference between the pHN409 and the pHN389 is only in that a region essential for autonomous replication is derived from pRE2895 or pRE8424. When the peptidase activity of PIP was compared between the cells of the *R. erythropolis* JCM3201 transformed with the pHN409 and the cells of the *R. erythropolis* JCM3201 transformed with the pHN389, there was almost no difference or otherwise, slightly higher activity in the cells transformed with the pHN409. Almost the same results were obtained when the *R. fascians* JCM10002 and the *R. opacus* DSM44193 were used as hosts. For all of the plasmids used, the peptidase activity of PIP in the *R. fascians* JCM10002 and the *R. opacus* DSM44193 was lower than the peptidase activity of PIP in the *R. erythropolis* JCM3201.

Next, the copy numbers of plasmids of the pNit-QC2 and the pNit-RC2 in the cell of *R. erythropolis* JCM3201 were investigated. The experimental approach conformed to the method by Projan et al. (Projan et al., Plasmid *9* 182-190 [1983]). This method requires knowing the genome size of the *R. erythropolis* JCM3201 in order to calculate the copy numbers of plasmids. According to van der Geize et al., the genome size of a *R. erythropolis* strain RG1, a strain derived from a *R. erythropolis* strain ATCC4277, is 6 megabase pairs (Mbp), and the *R. erythropolis* strain ATCC4277 and the *R. erythropolis* strain JCM3201 are nearly equivalent strains. Therefore, calculation was conducted with the genome size of the *R. erythropolis* strain JCM3201 as 6 Mbp. The results of the copy numbers were 47±5 for the pNit-QC2 and 64±5 for the pNit-RC2.

[Example 12]

Plasmid incompatibility

In most cases, heterologous plasmids having the same replication origin generally can not coexist in the cell of a bacterium. This is due to a phenomenon called plasmid incompatibility (Novick, Microbiol. Rev. *51* 381-395 [1987]), and there has been a report on plasmid incompatibility for *Mycobacterium*, the related genus of *Rhodococcus* (Stolt and Stoker, Microbiology *142* 2795-2802 [1996]). The present inventors have separated two endogenous plasmids (pRE2895 and pRE8424) having different sequences from *R. erythropolis*, and have therefore considered that several plasmids can be utilized in recombinant protein production by allowing them to coexist in a single cell. Thus, the plasmid incompatibility of the pTip and pNit vectors was initially investigated.

A first transformation of *R. erythropolis* JCM3201 was carried out with the pNit-QC2 or the pNit-RC2, and a second transformation of these transformant cells was conducted with the pNit-QT2 or the pNit-RT2. After the second transformation, each transformant was selected on a LB solid medium containing only tetracycline. The result is shown in Table 4. In Table 4, the second column from the right represents the number of colonies appearing on the solid medium containing tetracycline in the case that 1 μg each of the plasmid DNA was used in the second transformation. The rightmost column indicates the percentage (%) of the colony where the plasmid used in the first transformation remains after the second

transformation, that is, the incidence of colonies resistant to tetracycline after the second transformation. In this case, the number of colonies investigated is 20 colonies each (n=20). As shown in Table 4, the use of two plasmids having the same replication origin resulted in extreme reduction in the efficiency of the second transformation and frequent loss of the plasmid used in the first transformation after the second transformation and as such, can be said to cause incompatibility. By contrast, two plasmids having different replication origins did not reduce the efficiency of the second transformation and allowed the plasmids used in the first transformation to stably exist even after the second transformation. Thus, it has been suggested that the plasmids having different replication origins do not cause incompatibility. This means plasmids derived from pRE2895 and plasmids derived from pRE8424 are fully "compatible."

[Example 13]

Coexpression of recombinant proteins in single cell

As described in Example 12, the plasmids derived from pRE2895 and the plasmids derived from pRE8424 were fully compatible and could coexist in the single cell of *R. erythropolis*. From this point of view, the present inventors have attempted the coexpression of PIP and GFP in a single cell.

At first, primers represented by SEQ ID NO: 86 (sHN337) and 87 (sHN338) in the sequence listing were used to perform DNA amplification by PCR with the pHN187 (see Reference Example 1) as a template. The obtained 0.2-kb fragment contains the 5' end portion of the *GFP* gene. This fragment was digested with *Nco*I, and its 5' ends were phosphorylated. On the other hand, primers represented by SEQ ID NO: 88 (sHN339) and 89 (sHN340) in the sequence listing were used to perform DNA amplification by PCR with the pHN187 as a template. The obtained 0.5-kb fragment contains the 3' end portion of the *GFP* gene. This fragment was digested with *Bgl*II, and its 5' ends were phosphorylated. These two DNA fragments were simultaneously introduced into the *Nco*I/*Bgl*II sites of the pNit-QT1 and the pNit-RT1, respectively. The resulting plasmids were designated as pHN425 and pHN426, respectively. The pHN425 and the pHN426 contain the full-length *GFP* gene and are fused with a sequence for attaching a 6xHis tag to the C terminus of GFP.

Although the *Nco*I site present within the *GFP* gene is eliminated during the above-described procedures, the function of the GFP is not changed.

R. erythropolis JCM3201 was cotransformed with the pHN425 and the pHN389, and a cotransformant was selected on a medium containing both tetracycline and chloramphenicol. Alternatively, *R. erythropolis* JCM3201 was cotransformed with the pHN426 and the pHN409, a cotransformant was selected on a medium containing both tetracycline and chloramphenicol. As a control experiment, *R. erythropolis* JCM3201 was separately transformed with the pHN425, the pHN426, the pHN389, and the pHN409. These six types of transformants were allowed to express PIP and GFP as described in Example 1, which were in turn purified by metal chelate chromatography that employs a nickel ion. SDS polyacrylamide electrophoresis for the purification of the recombinant proteins and for the samples before and after purification was conducted by the following procedures. The 6xHis tag was attached to the C terminus of the PIP, and purification was performed using the Ni-NTA Superflow (manufactured by QIAGEN) according to the instruction.

Hereinafter, a specific purification method will be illustrated. Procedures for the purification were performed at 4°C. The bacterial cells (in 20 ml culture solution) where the protein was expressed were recovered and suspended in 1 ml of the NT-Buffer (50 mM Tris-HCl (pH 8.0), 100 mM sodium chloride, and 1 mM dithiothreitol), to which 1 g of glass beads (with a diameter of 0.105 to 0.125 mm) was then added. These beads were put into a reciprocating shaking motion at a speed of 6 m/sec for 20 seconds in the Fast-prep FP120 (manufactured by SAVANT) to destroy the cells. Following centrifugation at 20,000xg, 700 μl of the resulting supernatant was supplemented with the Ni-NTA Superflow equilibrated in advance with the NT-Buffer to bring the bed volume to 40 μl. While this was stirred by rotation for 1 hour, the Ni-NTA Super flow beads were bound to the protein attached to the 6xHis tag. These beads were washed four times with the NT-Buffer and then suspended three times in 120 μl of the NTE-Buffer (50 mM Tris-HCl (pH 7.0), 100 mM sodium chloride, 1 mM dithiothreitol, and 400 mM imidazole), thereby eluting the protein attached to the 6xHis tag from the beads. A 10-μl aliquot of the sample was subjected to 12% SDS polyacrylamide electrophoresis according to an ordinary method. A result of staining the gel with Coomassie

Brilliant Green G-250 after analysis by SDS polyacrylamide electrophoresis is shown in Figure 21.

In Figure 21, an odd-numbered lane indicates the crude extract of the cell (i.e., sample before purification), and an even-numbered lane indicates the sample purified by metal chelate chromatography. In addition, the drawing shows the samples from the *R. erythropolis* JCM3201 cotransformed with the pHN425 and the pHN389 (lanes 1 and 2) and with the pHN426 and the pHN409 (lanes 3 and 4) as well as the samples from the *R. erythropolis* JCM3201 transformed with the pHN425 (lanes 5 and 6), with the pHN426 (lanes 7 and 8), with the pHN389 (lanes 9 and 10), and with the pHN409 (lanes 11 and 12).

Two bands are seen in each of the lanes 2 and 4 in Figure 21. This has indicated that the PIP and the GFP were coexpressed in the single cell and purified. No significant difference was observed in the expression levels of the PIP and the GFP between coexpression (lanes 2 and 4) and independent expression (lanes 6, 8, 10, and 12).

Table 1 shows a list of plasmids used in Examples. Table 2 shows a list of strains used in Examples. Table 3 shows the transformation efficiency of the pNit-QC2 and the pNit-RC2 for *R. erythropolis* JCM3201, *R. fascians* JCM10002, and *R. opacus* DSM44193. Table 4 shows a result of the cotransformation of *R. erythropolis* JCM 3201 with the pNit-QC2, the pNit-RC2, the pNit-QT2, and the pNit-RT2.

Table 1

Table 1 Main plasmids used in the present invention

Classification	Designation of plasmid	Remarks	Source
Cryptic plasmids of *R. erythropolis*	pRE2895	Source of *RepAB* (cryptic plasmid isolated from *R. erythropolis* JCM2895)	Japanese Patent Application No. 2002-235008
	pRE8424	Source of *Rep* (cryptic plasmid isolated from *R. erythropolis* DSM8424)	This study
	PRE2893	Cryptic plasmid isolated from *R. erythropolis* JCM2893	This study
	PRE2894	Cryptic plasmid isolated from *R. erythropolis* JCM2694	This study
	PRE43200	Cryptic plasmid isolated from *R. erythropolis* DSM43200	This study
For identification of DSO and SSO of pRE8424	pHN267	*Kan*^{r} on pGEM 3Zf(+)	This study
	pHN317	*Rep*, DSO, IR I, IR II (SSO) on pHN267	This study
	pHN345	*Rep*, DSO, mutated IR I, IR II (SSO) on pHN267	This study
	pHN362	*Rep*, DSO, IR I, mutated IR II on pHN267	This study
	pHN363	*Rep*, DSO, mutated IR I, mutated IR II on pHN267	This study
	pHN322	*Rep*, DSO, IR I, IR II (SSO) on pHN267	This study
	pHN343	*Rep*, DSO, IR II (SSO) an pHN267	This study
	pHN344	*Rep*, DSO, IR I, IR II (SSO) on pHN267	This study
	pHN324	*Rep*, IR I, IR II (SSO) on pHN267	This Study
Source of *Rep* region for pTip- and pNit- vectors	pHN372	2.0-kb region originating from pRE8424 on pBluescript SK (+), *BamHI* site is eliminated	This study
pTip-vectors	pTip-QT1	P_{TipA}, *Tet*^{r}, *RepAB* (pRE2895), MCS type 1	This study
	pTip-QT2	P_{TipA}, *Tet*^{r}, *RepAB* (pRE2895), MCS type 2	This study
	pTip-RT1	P_{TipA}, *Tet*^{r}, *Rep* (pRE8424), MCS type 1	This study
	pTip-RT2	P_{TipA}, *Tet*^{r}, *Rep* (pRE8424), MCS type 2	This study
	pTip-QC1	P_{TipA}, *Chl*^{r}, *RepAB* (pRE2895), MCS type 1	This study
	pTip-QC2	P_{TipA}, *Chl*^{r}, *RepAB* (pRE2895), MCS type 2	This study
	pTip-RC1	P_{TipA}, *Chl*^{r}, *Rep* (pRE8424), MCS type 1	This study
	pTip-RC2	P_{TipA}, *Chl*^{r}, *Rep* (pRE8424), MCS type 2	This study
pNit-vectors	pNit-QT1	P_{Nit}, *Tet*^{r}, *RepAB* (pRF2895), MCS type 1	This study
	pNit-QT2	P_{Nit}, *Tet*^{r}, *RepAB* (pRE2895), MCS type 2	This study
	pNit-RT1	P_{Nit}, *Tet*^{r}, *Rep* (pRE8424), MCS type 1	This study
	pNit-RT2	P_{Nit}, *Tet*^{r}, *Rep* (pRE8424), MCS type 2	This study
	pnit-QC1	P_{Nit}, *Chl*^{r}, *RepAB* (pRE2895), MCS type 1	This study
	pNit-QC2	P_{Nit}, *Chl*^{r}, *RepAB* (pRE2895), MCS type 2	This study

	pNit-RC1	P_{Nit}, *Chl*^{r}, *Rep* (pRE8424), MCS type 1	This study
	pNit-RC2	P_{Nit}, *Chl*^{r}, *Rep* (pRE8424), MCS Type 2	This study
PIP expression vectors	pHN771	*6xHis-PIP* in MCS of pTip-LCH1	Japanese Patent Application No. 2002-235008
	pHN379	*6xHis-PIP* in MCS of pTip-RT1	This study
	pHN348	*6xHis-PIP* in MCS of pTip-QC1	This study
	pHN380	*6xHis-PIP* in MCS of pTip-RC1	This study
	pHN407	*6xHis-PIP* in MCS of pNit-QT1	This study
	pHN885	*6xHis-PIP* in MCS of pNit-RT1	This study
	pHN409	*6xHis-PIP* in MCS of pNit-QC1	This study
	pHN389	*6xHis-PIP* in MCS of pNit-RC1	This study
	pHN410	P_{TipA} and LG10-RBS of pHN380 were substituted into P_{Nit} and wild-type TipA-RBS, respectively	This study
	pHN387	LG10-RBS of pHN389 was substituted into wild-type RBS of TipA-RBS	This study
	pHN381	P_{Nit} of pHN389 was substituted into P_{TipA}	This study
GFP expression vectors	pHN425	*6xHis-GFP* in MCS of pTip-QT1	This study
	pHN426	*6xHis-GFP* in MCS of pTip-RT1	This study

Table 2

Main strains used in the present invention

Genus/Species	Strain	Source	Application
Rhodococcus erythropolis	JCM2895	Japan Collection of Microorganisms	Source of pRE2895
Rhodococcus erythropolis	DSM8424	German Collection of Microorganisms end Cell Cultures	Source of pRE8424
Rhodococcus erythropolis	JCM2893	Japan Collection of Microorganisms	Source of pRE2893
Rhodococcus erythropolis	JCM2894	Japan Collection of Microorganisms	Source of pRE2894
Rhodococcus erythropolis	DSM43200	German Collection of Microorganisms and Cell Cultures	Source of pRE43200
Rhodococcus erythropolis	JCM3201	Japan Collection of Microorganisms	Host strain to express recombinant proteins
Rhodococcus fascians	JCM10002	Japan Collection of Microorganisms	Host strain to express recombinant proteins
Rhodococcus opacus	DSM44193	German Collection of Microorganisms and Cell Cultures	Host strain to express recombinant proteins
Rhodococcus ruber	JCM3205	Japan Collection of Microorganisms	Host strain to express recombinant proteins
Rhodococcus rhodochrous	JCM3202	Japan Collection of Microorganisms	Host strain to express recombinant Proteins
Streptomyces coelicolor	JCM4979	Japan Collection of Microorganisms	Source of *dnak* transcription terminator
Escherichia coli	DH5α		General cloning
Escherichia coli	ER2508	New England Biolabs	Source of Kan^r

Table 3

Transformation efficiency of pNit-QC2 and pNit-RC2

	Host cell		
Plasmid	*R. erythropolis*	*R. fascians*	*R. opacus*
pNit-QC2	3.8×10^5	8.2×10^2	1.6×10^4
pNit-RC2	2.8×10^5	4.0×10^2	5.2×10^2

Table 4

Plasmid incompatibility for *R. erythropolis* strain JCM3201

Plasmid used in first transformation	Plasmid used in second transformation	Efficiency of second transformation	Percentage of colony where plasmid used in first transformation remains (%; n=20)
not used	pNit-QT2	3.2×10^5	-
pNit-QC2	pNit-QT2	2.0×10^3	50
pNit-RC2	pNit-QT2	1.3×10^5	100
not used	pNit-RT2	4.4×10^4	-
pNit-QC2	pNit-RT2	3.3×10^4	100
pNit-RC2	pNit-RT2	2.4×10^2	65

All publications, patents, and patent applications cited herein are incorporated herein by reference in their entirety.

Industrial Applicability

By using novel vectors of the present invention capable of replication in the rolling circle mode of replication, which are an expression vector capable of inducer-inducible expression of a foreign gene under a temperature condition of 4°C to 35°C in a bacterium belonging to the genus *Rhodococcus* and a vector capable of inducer-independent and

constitutive expression of a foreign gene in a bacterium belonging to the genus *Rhodococcus*, a foreign protein can efficiently be produced in a bacterium belonging to the genus *Rhodococcus*. Especially the use of a microorganism capable of proliferation even at low temperatures as a host microorganism makes it possible to express and produce a protein whose expression is difficult or impossible under a typical temperature condition suitable for the proliferation of a microorganism, that is, at moderate and high temperatures exceeding approximately 15°C. In addition, there is a remarkable feature in the expression plasmid vectors presented here. Stable co-transformants with at least two vectors can be obtained without causing plasmid incompatibility. For the co-transformation, one vector should carry a DNA region involved in rolling-circle mode of replication, and another one should carry a DNA sequence that originates from pRE2895 and is involved in autonomous replication of a plasmid. Furthermore, such co-transformation makes it possible to co-express foreign proteins in an identical microorganism, if the genes for co-expression are respectively integrated into different vectors.

Sequence Listing Free Text

SEQ ID NOs: 1 to 48: primer and linker sequences

SEQ ID NOs: 49 to 56: vector sequences

SEQ ID NOs: 57 to 89: primer and linker sequences

SEQ ID NO: 90: endogenous plasmid pRE8424 sequence

SEQ ID NOs: 91 to 106: vector sequences

SEQ ID NO: 107: modified *TipA* gene promoter sequence